University of Alberta

Quantification of the Tumour Burden in Multiple Myeloma

by

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Abstract

Multiple myeloma is a hematological malignancy characterized by an infiltration of clonal plasma cells in the marrow. While the tumour burden can be reduced by therapy, patients invariably relapse and succumb to the disease. Most prognostic factors, which are needed to determine disease severity and outcome, are indirect measures for tumour burden, and the level of marrow plasma cells has failed to associate with outcome. The work presented here examined the tumour burden in myeloma patients using SYBR Green I in a real-time quantitative PCR assay. To determine the number of clonal cells (VDJ%), the unique IgH VDJ rearrangement found in every myeloma cell was utilized as a molecular signature. Here, the VDJ% of bone marrow and peripheral blood taken from patients just prior to treatment was found to correlate with remission length, while the plasma cell number from matched marrow samples did not.

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List of Abbreviations

AlloSCT	allogeneic stem cell transplants
ASCT	autologous stem cell transplant
BM	bone marrow
BMMC	bone marrow mononuclear cell
BMPC	bone marrow plasmacytosis
BrdU	5-bromo-2-deoxyuridine
C	constant
C(T)	cycle threshold
CCR	clinical complete remission
CDR	complementarity determining regions
CR	complete remission
D	diversity
del13	deletion 13
Dex	dexamethasone
DSSS	Durie Salmon Staging System
EFS	event free survival
FISH	fluorescent *in situ* hybridization
HR	hazard ratio
Ig	immunoglobulin
IgH	immunoglobulin heavy
IMWG	International Myeloma Working Group
ISS	International Staging System

J	Joining
LDA	limiting dilution assay
LDH	lactate dehydrogenase
MGUS	monoclonal gammopathy of undetermined significance
MM	multiple myeloma
MCR	molecular complete remission
MRD	minimal residual disease
OS	overall survival
PC	plasma cell
PC%	plasma cell percent
PD	progressive disease
PBMC	peripheral blood mononuclear cell
PR	partial remission
RB1	retinoblastoma 1
RQ-PCR	real-time quantitative PCR
SD	stable disease
SG	SYBR Green I
Thal	thalidomide
Tm	melting temperature
TTD	time to death
TTP	time to progression
V	variable
VAD	vincristine adriamycin dexamethasone
VDJ%	VDJ percent

VGPR	very good partial remission
β2m	β2 microglobulin

I. INTRODUCTION

Multiple myeloma (MM) is a hematological malignancy that accounts for 1.3% of all newly diagnosed cancers in Canada (1). Generally defined, MM is a B-lineage malignancy of the bone marrow (BM) characterized by at least two of the following three features: an overgrowth of clonal plasma cells (PCs), monoclonal immunoglobulin in the blood and lytic bone lesions. The marrow houses most of this population providing a specific environment for the tumour to grow and to provide tumour resistance to therapy (2). To understand MM, normal B-cell development must first be described.

A. B-lineage immunoglobulin rearrangement

The defining process in B-cell development is the rearrangement of the immunoglobulin (Ig) heavy and light chain genes to form a functional molecule. This process results in a unique genomic rearrangement that is central to the molecular characterization of MM and understanding techniques used in this study.

The Ig heavy (IgH) genomic region is comprised of 27 D segments, 6 J segments and up to 44 functional V segments (3). These segments start to reassemble in the genome of pro-B-cells (Figure I-1). Each B-cell will undergo a different rearrangement (within the limits of segment copies). The VDJ region is then subjected to somatic hypermutation, a mechanism involving programmed mutagenesis that, combined with unique VDJ rearrangement, results in a diversity of more than 10^{14} different antigen receptors in humans (4). In a similar process, the light chain gene undergoes a VJ rearrangement and somatic hypermutation. The light chain protein eventually partners with the IgH chain to form a functional Ig with a unique receptor. This process is antigen driven in the germinal centers of lymph nodes. Epitopes are presented to

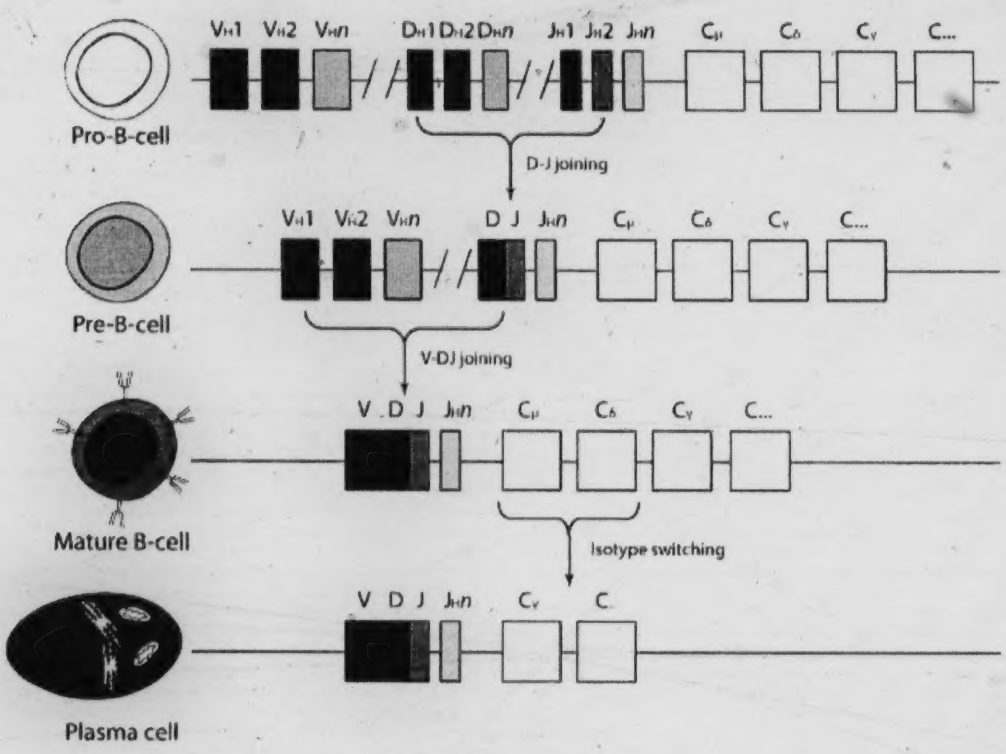


Figure I-1 Immunoglobulin Heavy Chain Rearrangement

The germ line DNA sequence of the immunoglobulin heavy chain (identical to all other human cells) contains all gene segments of the variable (V), diversity (D), joining (J) and constant (C) regions. As the B-cell matures from a pro-B-cell to mature B-cell (noted on the left) a D and J segment join, followed by a V segment. After this, the region undergoes hypermutation to increase diversity for antigen recognition. Finally, in normal plasma cells, the C region is switched to reflect the needed function of the immunoglobulin.

the developing B-cell to elicit an Ig protein with the needed specificity to recognize the foreign antigen.

The different segment rearrangements and hypermutations generate three regions, termed complementarity determining regions (CDR) 1, 2 and 3; which create the unique receptor for each Ig (5). Due to allelic exclusion, only one functional copy of the unique VDJ rearrangement can be found in the genome (6). In contrast, many copies can be detected in the

transcriptome and proteome of late stage B-cells. This unique rearrangement can be used as a biomarker for the clonal cells derived from the same B-cell.

Once a final rearrangement is made, a constant region, or class, at the base of the Ig protein is needed to facilitate different functions of immunoglobulin. By default, the Ig class expressed with newly rearranged cells is IgM. As the B-cell migrates out of the lymph node, it matures into a PC, a highly specialized cell that produces an abundance of Ig to effect an immune reaction. The PC is the final differentiation stage in the B-lineage. Through a process called class switching, a more appropriate Ig class is expressed by removing the unneeded isotype DNA upstream Figure I-1. There are five Ig classes: IgM, IgD, IgE, IgG and IgA.

MM cells have rearranged VDJ Ig genes that have also undergone somatic hypermutation and contain no intra-clonal variation in this Ig region (7). This disease is considered a "post-switch" cancer, in that most MM immunoglobulins have a constant region other than IgM, while other hematological cancers such as Waldenstrom's macroglobulinemia (8) and chronic lymphocytic leukemia (9) are considered pre-switch, expressing an IgM isotype. These cancers are derived from cells earlier in the B-cell development then MM. In a large study by the International Myeloma Working Group (IMWG), it was observed that 60% of MMs had IgG, 24% had IgA, 11% produced only a light chain, 3% had IgD and 2% had a biclonal or other isotypes (10). The survival expectancy for patients with an IgA MM is shorter than for those with an IgG disease (40-month median compared to 49-month median), while those with expression of only light chain have a median survival of 35 months (10).

MM is monoclonal, with the same unique Ig gene rearrangement in each clonal cell. As such, PCR primers can be designed to amplify the unique sequence within CDR1, 2 or 3 regions, specific to the clone (often termed allele specific oligonucleotides, ASOs). PCR amplification

using these patient specific primers will only occur if the target sequence from the clone is present. As a single copy of the rearrangement is present each clonal cell, the number of clonal cells in a population can be determined using quantitative PCR techniques that can identify the number of targets added into a PCR reaction.

B. Clonal cell types in MM

For a clinical diagnosis of MM there must be at least 10% PCs in the marrow. However, other B-lineage cell types have been shown to harbour the clonal rearrangement in MM patients. The plasmablast, a precursor to the PC, can be found in the PB of MM patients and its presence is associated with a very poor outcome (11). B-cells clonally related to the BM PC population have been identified (12). In fact, late-stage B-cells, pre-B-like cells, pre-switch B-cells and memory B-cells (post switch cells that remain after the introduction of the antigen to elicit a quick immune response upon further exposure) containing the clonal IgH sequence have been identified in patients with MM (13, 14). Studies have identified clonotypic B-cells in both the BM (12, 14-19) and PB (18-24). There is some controversy whether the MM clone contains a population of cells expressing CD34, a marker that typically identifies hematopoietic stem cells. Two groups identified clonal sequences in a population of $CD34^+$ cells (25, 26). In contrast, others failed to detect these cells in the PB, BM or PB stem cell harvests (23, 27-29). These latter studies analyzed only a small number of cells and the unique $CD34^+$ population may have been missed. A clonal $CD34^+$ MM cell most likely would not be an originating hematopoietic stem cell, as Ig rearrangement has not yet occurred at this stage in hematopoietic lineage differentiation, but likely a clonal cell that expresses the CD34 marker. There is also controversy about the frequency of clonal cells in the B-cell compartment in PB: some groups report low levels of clonal cells in the PB while others report high frequencies (30).

C. Etiology of myeloma

The etiology of MM is, to date, unknown, and is debated among researchers. The central issue focuses on the cell type that sustains the tumour. A cancer stem cell is most likely responsible for the tumour growth, as oppose to general tumour cell proliferation, as MM PCs have a low growth rate/rare mitotic figures (31) and are not self-maintaining (32). Much debate is over the role of clonotypic B-cells in the malignancy. In 1996, Pilarski *et al.* (33) suggested four 'plausible' roles for the clonal B-cells:

> "1) Non-malignant clonal relatives of the MGUS that gave rise to myeloma. 2) Migratory malignant progeny of the myeloma clone that mediate cancer spread. 3) Malignant stem cells with potential for self-renewal. 4) A monocytoid/macrophage-like differentiation fork within the malignant clone."

Many believe that clonal B-cells are simply a non-malignant remnant of the tumourigenic process, and a PC is responsible for the growth and maintenance of MM. One paper utilized a SCID-hu murine model to identify the proliferative compartment in MM (34). The authors observed the outgrowth of myeloma cells in mice from the PC population but not from the PC-depleted population. However, this model utilized a human embryonic bone fragment for myeloma growth *in vivo*, which does not reflect the nature of the aged bone microenvironment. As such, the embryonic bone may support the growth of certain cell types while inhibiting others that may grow in aged bone marrow. When MM patients were treated with an antibody directed against B-cells, only a single objective response was observed in two trials (35, 36). This failure might suggest that B-cells have a passive role in the maintenance of the malignancy. Alternatively, as the PC compartment was not targeted with the therapy, the poor responses noted may be due to the low levels of PC apoptosis (37).

Another possible etiology suggests that an aspect of the clonal B-cell compartment is responsible for the growth and maintenance of the malignancy. Importantly, clonal B-cells have

been observed after high dose therapy (24, 38) suggesting a possible and plausible role in relapse and thus disease development. It has been observed that the non-PC population from an MM cell line has stem-like properties (39); however this assumption was made without clonal identification of these populations. It has also been suggested that a population of B-cells that express the stem cell marker $CD34^+$ mediate disease expansion. In Pilarski and Belch (40), $CD34^+$ enriched populations were able to grow MM in immunodeficient mice. No PCs were observed in this population but exhaustive tests to show conclusively that no PCs were present were not done. Guikema *et al.* discuss an etiology where a memory B-cell expands to initiate the disease (41). This hypothesis suggests that only a subset of clonal B-cells contribute to the malignancy: most clonal B-cells are part of the tumourigenic process while the memory B-cell mediates initial disease and relapse. The most likely hypothesis is one suggested by Pilarski *et al.* (13), that MM is a multifactorial disease involving many different cells that may be initiated and maintained though different pathways.

D. Multiple myeloma: the disease

MM can start *de novo* or evolve from monoclonal gammopathy of undetermined significance (MGUS), an asymptomatic monoclonal gammopathy, at a rate of 1% per year (42). While MM responds to therapy, the ultimate eradication of the disease remains unachievable. Relapses are frequent, occurring on average every 24 months, with patients succumbing to the disease on average in 4.4 years (43).

1. Clinical characteristics

The most identifying characteristics of MM include high levels of clonal PCs, osteolytic lesions and detectable serum M-component proteins (44). The number of abnormal PCs generally defines the difference between MM and MGUS, where greater than 10% PCs defines

MM and less than 10% defines MGUS. Osteolytic lesions are common but not universal in MM, with about 80% of patients having bone involvement at diagnosis (45). Due to an increase in osteoclastic activity in MM, bone is resorbed leading to bone softening, bone pain and hypercalcemia (46). As the BM can be overloaded with PCs, immunoglobulin generated and excreted by PCs as a part of their normal function is released into the peripheral circulation. This high level of circulating protein can have negative effects on the kidneys and many patients succumb to the disease by way of kidney failure.

Some patient and tumour characteristics have relevant associations with survival. These are able to predict either event free survival (EFS), defined as the time until relapse, or overall survival (OS), defined as the time to death. In an incurable cancer, factors that grade tumour aggression and acquired drug resistance are essential to informing clinicians and patients about the disease and treatment options. San Miguel *et al.* (47) notes three essential roles of prognostic factors and staging:

> 1: to provide more individualized information about the disease outcome, something systematically required by the patients and their families;
>
> 2: to identify risk groups in order to adapt patient treatment according to the expected outcome and to compare the results between different therapeutic strategies;
>
> 3: to discover associations between biological features of the tumor clone and clinical behavior in order to better understand the pathogenic background of the disease.

Many factors are indirect measures of disease burden, while others define specific tumour phenotypes. These factors can predict survival and can lead clinicians to better-informed treatment decisions. Additionally, combinations of individual factors have been

utilized to improve the prediction of patient survival. Below is a brief discussion of some of these factors.

a) β2 microglobulin

β2 microglobulin (β2m) is one of two factors currently used by many clinicians to stage MM and is perhaps the most important measurement in MM. This protein is produced in nearly every human cell and is associated with the MHC class I molecule to aid in cellular immunity. β2m is presumably shed from the cell surface into the blood (48). In patients with MM, levels of peripheral β2m can be above that of healthy individuals because of elevated cellular numbers due to the tumor burden and poor peripheral clearing because of renal function deterioration. High levels of β2m (generally >3.5g/l) have been shown to have prognostic significance at both the diagnostic and plateau phase (49-51).

b) Serum albumin

The second measurement often used to stage MM is serum albumin. This protein circulates in the PB maintaining normal osmotic pressure within the body. In mild forms of MM, albumin levels are close to normal; however, in aggressive forms, the amount of albumin is reduced relative to normal individuals (<30g/L) (10, 52).

c) LDH

Lactate dehydrogenase (LDH) is an enzyme present in most organisms and is associated with tissue destruction, particularly hemolysis. In MM, it was discovered to be a prognostic indicator where high levels before or by 2 weeks after therapy correlated with a poor prognosis (53). These high levels were associated with aggressive disease and a 1-year survival rate of 5%.

d) Calcium

Levels of serum calcium have long been associated with outcome in MM. Elevated calcium in MM is caused by widespread tumor-induced bone destruction through osteoclast activation and can reflect a proportional tumour burden level (54). Used as part of the Durie Salmon Staging system (I.D.1.l), elevated calcium is associated with poor outcome (55).

e) Creatinine

Creatinine is a metabolite that is cleared by normally functioning kidneys. During renal dysfunction, creatinine is built up in the blood due to reduced clearance and thus, an elevated level in MM patients reflects impaired kidney function. This measure is moderately linked to tumour mass and significantly correlated with survival (55).

f) Hemoglobin

Hemoglobin levels are a reflection of red blood cell counts. In MM, the tumour mass can impair blood production in the BM. Low levels of hemoglobin indicate anemia, an identifying characteristic of MM (44), and poor prognosis (55). Additionally, levels of hemoglobin have been shown to correlate with tumour burden (55), likely a result of the inverse relationship between tumour occupation of the BM and the space available for normal hematopoesis.

g) Serum free light chain ratio

A normal population of B-cells produces free lambda and kappa light chains that are never bound to the heavy chain to form a functional immunoglobulin. A constant ratio of freely associated lambda to kappa light chains is found in the serum. In patients with MM, the clonal PCs can excrete an excess of one type of light chain that alters this ratio. Abnormal serum free light chain concentrations have been noted in light chain MM (56), non-secretory MM (57) and

MM with intact immunoglobulin (58). The free light chain ratio has been shown to be an independent prognostic factor that correlates with survival (59) most likely due to a proportional association with tumour burden.

h) Cytogenetics

MM is a cancer involving cells of the B lineage that have undergone an immunoglobulin rearrangement. During this rearrangement, the germline DNA is broken to reassemble a unique and functional immunoglobulin. A consequence in MM is that translocations may occur at sites of these breaks. Additionally, MM is considered highly unstable, resulting in other gross chromosomal changes such as amplifications, deletions and ploidy changes. MM cells are generally characterized as having one of two chromosomal patterns: hyperdiploidy, or non-hyperdiploidy with recurrent IgH associated translocations. These two phenotypes are typically mutually exclusive and each occurs in approximately half of all MMs (60).

Chromosome 13 deletions (del 13) are the most common chromosomal alteration in MM. Using interphase fluorescent *in sito* hybridization (FISH), del 13 occurs in 75-90% of PCs in 30-55% of all MM patients (60). The deletion of one copy of chromosome 13 was more prevalent in aggressive stage MMs, and associated with a shorter EFS and OS (61). However, some debate surrounds whether del 13 is directly responsible for a poorer outcome or is simply a product of the general hypodiplody (60). Partial deletions of chromosome 13 have also been observed. Specifically, a loss of 13q14 was seen in 15% of those cases that were also classified as del 13 by interphase FISH (60).

Other chromosomal changes have been associated with outcome. Translocations involving chromosome 14 (IgH locus) such as t(4;14) and t(14;16) and deletion of the p53 and RB1 genes are linked with poor survival (47, 62). In contrast, t(11;14) (63) and trisomy 6, 9, and

17 (62) have better outcomes. Deletions of the retinoblastoma 1 gene sequence (or RB1) as detected by FISH are hard to analyze as the gene locus harbouring the RB1 gene is in the commonly deleted region 13q14 and may not indicate poor survival but a deletion of chromosome 13. One study showed a significant correlation between RB1 gene deletion and chromosome 14 translocations, indicating that RB1 deletions may reflect effects from important translocations (64). Additionally, this study showed that patients with an RB1 deletion as a sole abnormality displayed the same prognosis as patients without any abnormalities, suggesting that RB1 deletions may not be informative. Prognostically significant alterations of chromosome 1 have also been identified. Amplifications of the q arm and deletions of the p arm of chromosome 1 are frequently observed (65). Expression changes that reflect these alterations (66) and deletions of the p arm (67) have been associated with poor outcome.

i) Gene mutations and expression alterations

In addition to gene deletions, simple mutations or expression changes can be associated with outcome. Like in many other cancers, mutations activating oncogenes or deactivating tumour suppressor genes can be found in MM. For example, mutations leading to the inactivation of p53 or Rb are linked to aggressive disease (68). Unlike most cancers, mutations in p53 are considered a late event (69). Activating mutations in the oncogene K-*ras*, which regulates proliferation and differentiation, and methylation changes of p16 are correlated with poor prognosis, although the latter is directly linked to highly proliferative PCs (70, 71).

j) Age

The age of diagnosis has been a long-standing factor of survival. Patients younger than 60-70 have prolonged disease survival (47, 72, 73). Longer survival was observed in young patients (<40) when compared to patients of all ages (74). However, it remains to be seen if

these correlations are dependent on other factors. In younger patients, more aggressive therapies can be used that may lead to longer survival, as the general health of the individual is better. Alternatively, older patients would be less able to tolerate more aggressive treatments, and thus, less likely to receive or stay on these therapies.

k) Labeling index

Many of the factors noted above deal with measuring the tumour burden. In contrast, the labeling index is a unique measure of proliferation, independent of tumour burden (75). Studies using tritiated thymidine autoradiography or 5-bromo-2-deoxyuridine (BrdU) showed a correlation between high nucleotide incorporation and poor outcome (75, 76). These tests analyze the amount of DNA incorporation and as such indicate DNA replication or repair. The PC labeling index is considered one of the most important prognostic factors with a large difference in median survival between patients with high, medium and low PC proliferation. This method, however, can produce ambiguous results as cells undergoing DNA repair also incorporate BrdU (77, 78). An alternative method to nucleotide incorporation is S-phase analysis, which is based on the incorporation of propidium iodide into DNA. The percentage of PCs in S-phase can be measured by analyzing the number $CD138^+$ cells (a PC marker) with DNA content between 2N and 4N (which represent G1 and G2, respectively). This method established that a high number of proliferative PCs correlates with poor survival (79). However, S-phase analysis also can generate ambiguous results. Pilarski *et al.* (80) have shown that hyperdiploid cells can be misinterpreted as S-phase cells, confounding results. Therefore, while much data identifies that proliferating PCs in MM patients can predict poor survival, these techniques that measure proliferation are flawed and may be partially or totally artifactual.

l) Durie Salmon staging method

In the 1970s, Durie and Salmon (55) created a staging method that incorporated various factors for MM:

Stage I

All of the following:

- Hemoglobin value > 10 g/dL
- Serum calcium value normal (< 12 mg/dL)
- On roentgenogram, normal bone structure or solitary bone plasmacytoma only
- Low M-component production rates
- IgG value < 5 g/dL
- IgA value < 3 g/dL
- Urine light chain M-component on electrophoresis < 4g/24 h

Stage II

Overall data as minimally abnormal as shown for stage I, and no single value as abnormal as defined for stage III.

Stage III

One or more of the following:

- Hemoglobin value < 8.5 g/dL
- Serum calcium value > 12 mg/dL
- Advanced lytic bone lesions
- High-M-component production rates
- IgG value > 7 g/dL
- IgA value > 5 g/dL
- Urine light chain M-component on electrophoresis > 12g/24 h

Subclassification

A = relatively normal renal function (serum creatinine value < 2.0 mg/dL)

B = abnormal renal function (serum creatinine ≥ 2.0 mg/dL)

The Durie Salmon staging system (DSSS) has been used for many years; however, limitations make this system difficult to use. The system is good only for staging patients at diagnosis. Further, the radiographs were hard to read, it was difficult to consistently grade

'advanced lytic bone lesions', and the system failed to prove prognostically reliable in patients treated with high dose therapies (defined below).

m) International Staging System

Many years after the introduction of the DSSS a newer system was developed. The new International Staging System (ISS) was designed to reduce confusion from results and be applicable to all geographic regions and ages (10). Additionally, the ISS is able to divide patients into more evenly distributed categories, which are more useful for judging treatment options. This model uses only two factors to stage patients, β2m and albumin:

Stage I

- β2m <3.5 mg/L
- albumin >35 mg/L

Stage II

- Not I or III

Stage III

- β2m >3.5 mg/L

While the ISS has made staging MM easier, some suggest this system still only judges the tumour load (48). Other factors that measure cell kinetics and genetic information to identify basic biological information about the tumour that is useful for determining prognosis are omitted in this system.

2. Treatment of MM

Up until the last decade, little had changed in the treatment of MM. Melphalan and prednisone was a standard therapy for many years. It was later revealed that higher doses of melphalan could effect a better responses (81). Many studies showed that high dose therapy followed by autologous stem cell transplant had promise as an even better treatment. In a

study comparing conventional dose therapy to high-dose therapy combined with autologous transplant, the latter was shown to have a better response rate and longer event-free and overall survivals (82). In recent years, a surge of new, biologically based therapies has been developed. With these new treatments, patient toxicity can be reduced, but these therapies continue to produce only short-term tumour responses. In contrast to chemotherapeutic agents that generally result in cytotoxic death, biologically based therapies activate or inhibit cellular or system functions to effect tumour death.

a) Response

Evaluating the tumour response to therapy is important. So too, is identifying the treatment regimens that effect the most and/or best responses. A good tumour response is usually coupled with less disease related effects. In MM, response is best defined by the reduction in BM PCs and/or a reduction in the serum M-protein levels. Other factors that correlate with tumour burden can also be used, but remain largely undefined for quantifying response. Clinicians and researchers use the level of response or the number of responses as a comparative measure between different treatment regimens. There is a significant correlation between response and survival (83, 84), although kinetics and degree of response were not shown to be significant. That is, patients who responded to therapy had a longer survival then those that do not respond, but the amount of tumour reduction did not correlate with survival.

The IMWG has published a standardized set of criteria for grading response (85). They are:

1) Complete response (CR) is defined as "negative immunofixation on the serum and urine and disappearance of any soft tissue plasmacytomas and ≤5% plasma cells in the bone marrow"

2) Very good partial response (VGPR) is defined as "serum and urine M-protein detectable by immunofixation but not on electrophoresis or 90% or greater reduction in serum M-protein plus urine M-protein level <100mg per 25hs"

3) Partial response (PR) is defined as "≥50% reduction of serum M-protein and reduction in 24-hr urinary M-protein by ≥90%"

4) Stable disease (SD) can be defined as being a lesser response than PR but having at least a 5% decrease in the serum or urine M-protein

5) Progressive disease (PD) is defined as anything else.

Below are described the drugs and therapies given to patients from whom samples were taken for this study.

b) Vincristine

Vincristine is a widely used chemotherapeutic drug derived from the periwinkle. One of three combination drugs commonly given to MM patients, with doxorubicin and dexamethasone (VAD), vincristine is a vinca alkaloid that inhibits mitosis by blocking tubulin formation. In many cases, VAD is used as induction or front-line therapy in younger myeloma patients who will receive a stem cell transplant. The drug combination does not contain alkylating agents, which can introduce mutations, thus resulting in potentially mutated stem cell for transplant. The major drawback of this therapy is that vincristine can inhibit the growth of or kill non-tumour cells. However, it was found that a higher number of stem cells were preserved after treatment with VAD then with other therapies (86).

c) Doxorubicin

Alternatively named adriamycin, doxorubicin is a DNA intercalating drug. This chemotherapeutic agent binds DNA and causes the inhibition of helicases, blocking replication. Treatment with doxorubicin can lead to low blood counts and hair loss.

d) Dexamethasone

Dexamethasone (Dex) is an anti-inflammatory drug often used to balance effects of other therapies. In combination with vincristine and doxorubicin, VAD, Dex is one of the most used treatments for newly diagnosed MM with a 55% response rate (87). Dex is the most active component of VAD and can be used alone as a frontline therapy or in treatment of relapsed MM. It was noted that physicians are using Dex alone or with thalidomide for induction therapy (88). Thalidomide/Dex has been shown to significantly effect more response than VAD as a frontline therapy, although less stem cells were obtained from harvests after thalidomide/Dex treatments (89). In older patients ineligible for transplant, Dex extended the remission duration when used as a maintenance therapy, but did not improve overall survival (90). The percentage of patients who developed deep venous thrombosis increased with the Dex dosage (88, 91).

e) Thalidomide

Originally designed as a sedative, thalidomide (Thal) gained historic recognition in the 1950s for causing birth defects in the children born to women who took the drug during pregnancy. Thal has seen a resurgence as a therapeutic drug for MM (92). While Thal is known to have anti-angiogenic effects, the specific mode of action in MM is not fully understood. The proposed mechanisms include the inhibition of tumour necrosis factor α, a reduction in essential MM microvessel density, an increase in cell-mediated cytotoxic effects and an alteration of the expression of cellular adhesion molecules (93, 94). Thal has been used in combination with Dex for front-line therapy and had a tumour response rate of 64% (94, 95). Thal is also being

explored as a maintenance therapy: low doses are provided to patients in remission in efforts to extending remission durations (96).

f) Lenalidomide

Lenalidomide, or Revlimid, is an immunomodulatory derivative of thalidomide, and it was developed to be more tolerable and effective than thalidomide. It was shown to be more effective than Thal in *in vitro* tests (97). Side effects such as sedation and neuropathy, which afflict patients treated with Thal, are not observed in patients treated with lenalidomide; however, myelosuppression was frequent. Lenalidomide was tested in MM patients with numerous relapses or those who were refractory to other treatments and the results revealed an overall response rate of 25% (98). In a clinical trial of lenalidomide and Dex for front-line therapy, 91% of patients had at least a partial response (99).

g) Bortezomib

One of the most novel agents for cancer therapy, bortezomib, or Velcade, has become an increasingly popular MM therapeutic. This biological agent has a very unique molecular composition, contains a boron atom that binds to the catalytic site of the 26S proteasome (100). Cell death is effected by disrupting the ubiquitin-proteasome pathway, which is involved in the degradation of cellular proteins. If this pathway is disrupted, there is a build-up of pro-apoptotic and cell cycle regulating proteins that eventually lead to apoptosis (94, 101). Specifically, bortezomib has been shown to cause an upregulation of pro-apoptotic factors, downregulation of NF-κB and a reduction in the expression of transcripts for molecules involved in cell cycle, growth, survival and drug resistance. In frontline therapy, bortezomib/Dex has shown a response rate of 67-82% (102, 103). For relapsed/refractory MM, bortezomib had a

partial/complete response rate of 38% versus 18% in Dex treated patients (104). This response rate is low, likely because the MM may also be refractory to aspects of drug's mechanism.

h) Melphalan and prednisone

Melphalan, a nitrogen mustard alkylating agent, and prednisone, an immunosuppressing corticosteroid, have been used since the 1960's to treat MM (105). As newer therapies were introduced, this regimen was less frequently used in younger patients. While the response rate is low, survival is comparable to other therapies (106). Patients ineligible for a stem cell transplant, mainly those older than 65-70, are generally treated with this regimen. In recent years, melphalan and prednisone have been combined with Thal for treatment of MM in elderly patients. This regimen showed improved response rates, progression free survival (107) and overall survival (108). Additionally, melphalan and prednisone have been combined with bortezomib (109) or lenalidomide (110), also with superior results. Standard therapy in younger patients involve treatment with non-alkylating agents (VAD) to reduce the tumour burden, following which, hematopoietic stem cells are harvested. Once a population of stem cells is removed, high dose melphalan can then be used to reduce the tumour load further, before the stem cells are transplanted back into the patient.

i) Cyclophosphamide

Cyclophosphamide is another nitrogen mustard alkylating agent that kills tumour cells by introducing DNA damage, and is commonly used in combination with other drugs. In contrast to the paradigm noted above, where alkylating agents are not used prior to stem cell harvests, cyclophosphamide can be used for induction therapy. The Nordic Myeloma Study Group showed equal stem cell recovery, tumour response rates and progression free survival between induction therapy with VAD verses cyclophosphamide/Dex (111). The advantage is

that patients treated with cyclophosphamide/Dex experienced less toxic effects otherwise resulting in premature mortalities. Additionally, cyclophosphamide does not require burdensome central venous lines for administration.

j) LymphoRad

LymphoRad is a radioiodinated B-lymphocyte stimulator that binds to B-cells, delivering a dose of radioactivity to each cell. An inverse correlation was found between high levels of B-lymphocyte stimulator and poor overall survival (112). There are current ongoing Phase 1 trials using LymphoRad.

k) Stem cell transplants

Compared to conventional chemotherapy, stem cell transplants, while not curative, greatly improve the response rate, event free survival, and overall survival of MM patients (82, 113). The most common stem cell transplant in MM is a self or autologous stem cell transplant (ASCT). Non-alkylating therapies are used to reduce the tumour burden, following which, hematopoietic stem cells are mobilized from the BM to the PB and collected. After further tumour elimination, the stem cells are reinfused into the patient. This regimen results in increased responses and prolonged EFS and OS (114). Transplants have been extensively proven to be beneficial in younger patients (typically less than 65 years old) (91) while older patients have generally been excluded from these trials. The side effects, such as risk of infection and hematopoietic cell loss associated with an ASCT are often considered too great to benefit older patients; however, some studies suggest that improving supportive care may make ASCT more feasible for older patients (115). While autologous transplants are beneficial, there is one major flaw: the reinfusion products contain biologically relevant malignant cells (116) that have been proposed to mediate post transplant relapse (40). Purging involves the purification of the stem

cell harvests by selecting only the stem cells or by removing non-stem cells to reduce or eradicate the number of tumour cells to be engrafted. Some data indicate an improved outcome in patients transplanted with purged grafts; however, in most cases the tumour cells are not fully eradicated from the harvest samples (117).

Allogeneic stem cell transplants (AlloSCTs) differ from ASCTs in that the patients are engrafted with hematopoietic material from an HLA-matched donor. It has been shown that AlloSCTs are superior to ASCTs as they result in higher response rates and longer remissions (118). The efficacy of the AlloSCT is in part due to two factors; 1) no tumour cells are being reinfused into the patient and 2) graft versus myeloma effect (119, 120). The graft versus myeloma effect *could* permanently eradicate the myeloma tumour (121) in that the transplanted immune cells can target and kill tumour cells. However, transplanted immune cells can also kill normal, healthy cells, termed graft versus host disease, resulting in a mortality rate for AlloSCT of 40-50% (122). With the additional difficulty of finding HLA-matched donors, AlloSCT are performed infrequently.

Patients relapsing from an AlloSCT are eligible for a donor-lymphocyte (leukocyte) infusion where additional cells from the same donor are given to the patient. A study involving 25 MM patients receiving a donor-lymphocyte infusion showed anti-tumour activity, however, there were clear limitations to the therapy as graft versus host disease still occurred and there was no response in a significant number of patients (123).

A newer technique involves a mini AlloSCT with non-myeloablative chemotherapies that does not fully destroy the BM before undergoing the transplant. This therapy has been shown to offer good MM control but patients still can develop graft versus host disease (124).

E. Disease burden and minimal residual disease

Measuring the disease burden in any malignancy is essential to determine the disease severity, tumour response to therapy and identifying relapse. For solid tumours, size and/or volume determines burden. In hematological malignancies, the tumour cells intermingle with normal cells in blood and marrow. Tumour cells are generally not distinguishable or observable unless they are crowded in the marrow and identified pathologically. Even yet, MM is defined by presence of greater than 10% PCs in the marrow and not the occurrence of tumour cells. The extent of bone marrow plasmacytosis (BMPC) has been used for decades to enumerate the number of PCs in the BM. The BMPC counts PCs, reported as a percentage, and conveys the volume of tumour infiltration. This measure, however, does not correlate with outcome (125), thus, measures that are more accurate must be developed to evaluate the tumour load.

MM can be treated to reduce tumour burden but the same clonally identical cancer eventually returns, indicating that residual tumour cells remain after treatment to regenerate the tumour. As such, many studies aim to evaluate the level of minimal residual disease (MRD). Tumour load during remission can be used to predict the time to relapse.

In MM, the main technique for detecting and measuring MRD is PCR. The unique VDJ rearrangement can be used in techniques that specifically identify clonal cells. These clonal populations are usually identified within a background of normal PB mononuclear cells or BM mononuclear cells.

1. Qualitative PCR for determining minimal residual disease

There are two types of MRD testing. The first is a basic qualitative analysis. Using PCR, the presence or absence of the clonal VDJ in a patient sample can be found. Typically, qualitative PCR is a nested PCR reaction, which is a two stage PCR where amplification product

from the first stage is used as template for the second reaction. Primers internal to those in the first stage reaction are used in the second. Nested reactions have much higher sensitivity than a standard PCR reaction as fewer targets are needed to generate a positive result. Qualitative PCR categorically defines patient samples as "yes", tumour cells are present, or "no", tumour cells are not present, based on a very sensitive detection level (10^{-6}, or 1 in 1 000 000 cells that can be detected) (126). A negative nested-PCR result in the BM aspirate defines a patient as being in molecular complete remission (MCR). In one study, only 27% of the 44 patients in clinical CR (CCR) after ASCT or AlloSCT were also in MCR (127). The patients in MCR had a longer relapse-free survival than those who did not achieve MCR. In patients who achieved a CCR after AlloSCT, 33% had a durable MCR and a five year survival of 100% while 27% with a durable positive BM PCR results and a 0% five year survival rate (128).

An MCR after ASCT is rare (15% (129)) and as standard chemotherapeutic or biologically based treatments are less effective then ASCT, a MCR would be even rarer after these treatments. Qualitative PCR thus is limited by the number of patients who are eligible for catagorization.

2. Quantitative techniques to measure tumour burden

The second and more informative method for determining MRD is to use quantitative measures. There are three main laboratory assays used to quantify tumour burden in MM. The first method is real-time quantitative PCR (RQ-PCR) for the unique clonal VDJ rearrangement. The second is the predecessor of RQ-PCR; a limiting dilution assay where the VDJ rearrangement is used to quantify the number of tumour cells. Flow cytometry for MM PCs is the third quantitative technique discussed here.

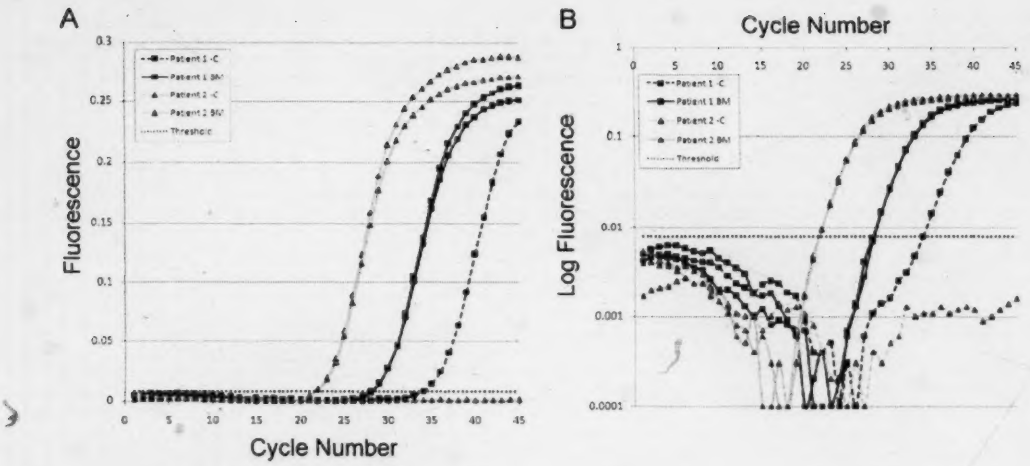


Figure I-2 RQ-PCR Amplification

Graphs depicting the relative (A) and log fluorescence (B) amplification of VDJ two patients' BM samples in duplicate with water controls. An increase in the VDJ amplicon, measured using fluorescence, is seen for two patient samples (NOR and MEB) and their negative controls (-C).

a) RQ-PCR

As with qualitative PCR, RQ-PCR requires that the clonal VDJ sequence be derived in order to generate unique primers. In contrast to qualitative PCR, which simply confirms the presence or absence of clonal cells in a sample, RQ-PCR determines the number of clonal cells using quantitative PCR. A standard PCR is performed until amplification saturation, but the final level of amplicons does not reflect of the starting level of targets. RQ-PCR, however, is a PCR method that employs fluorescent markers to track the accumulation of amplicons *during* the exponential amplification of the target sequence. The number of cycles required to reach the exponential phase of amplification is directly correlated to the number of original targets (Figure I-2). This cycle number is determined using the cycle threshold (C(T)), a line set above background fluorescence. Ririe explains it this way: "...fluorescence is acquired once each cycle, [and] the increase above background fluorescence begins at a cycle number dependent on [the]

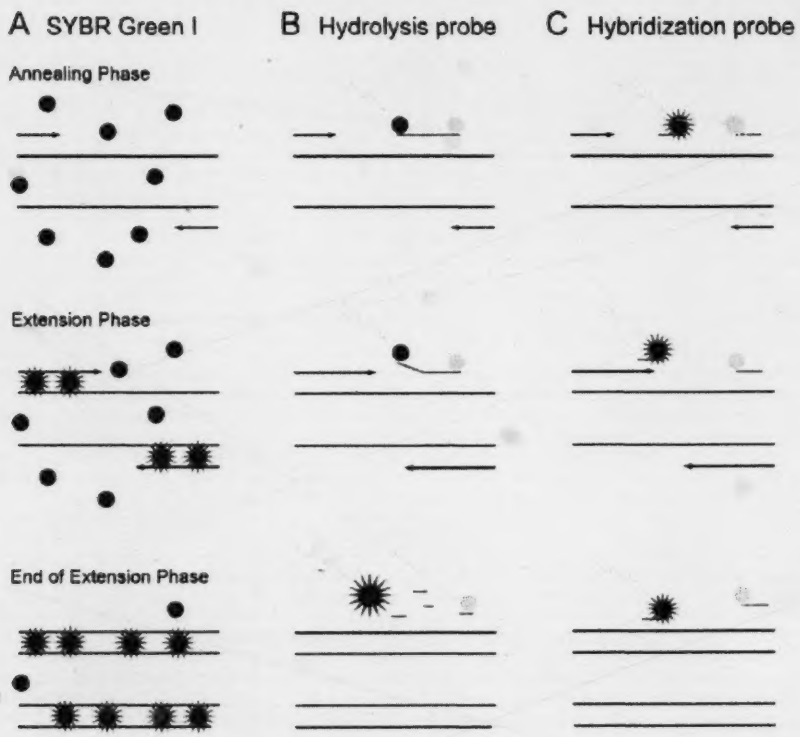


Figure I-3 Principles of Three RQ-PCR Techniques

(A) The fluorescence of SYBR Green I (SG) greatly enhanced upon binding to double-stranded DNA. During the extension phase, more SG will bind to the amplicon, resulting in increased fluorescence. Each PCR cycle accumulates more amplicon and more SG fluorescence. (B) The hydrolysis probe is conjugated with a reporter and quencher fluorochrome. Upon amplification, the hydrolysis probe is displaced and the reporter and quencher fluorochrome separate. This disjoining removes the quencher thereby allowing detection of the reporter fluorochrome. After each PCR cycle, the level of reporter accumulations is measured. (C) In a hybridization probe system, two separate probes are brought together during the annealing phase if the correct amplicon is present. The emission of the donor fluorochrome excites the acceptor fluorochrome. Emissions of the acceptor fluorochrome are measured.

Picture adapted from van der Velden *et.al*, 2003, Leukemia.

initial template concentration" (130). Three main RQ-PCR methods will be explained here (Figure I-3).

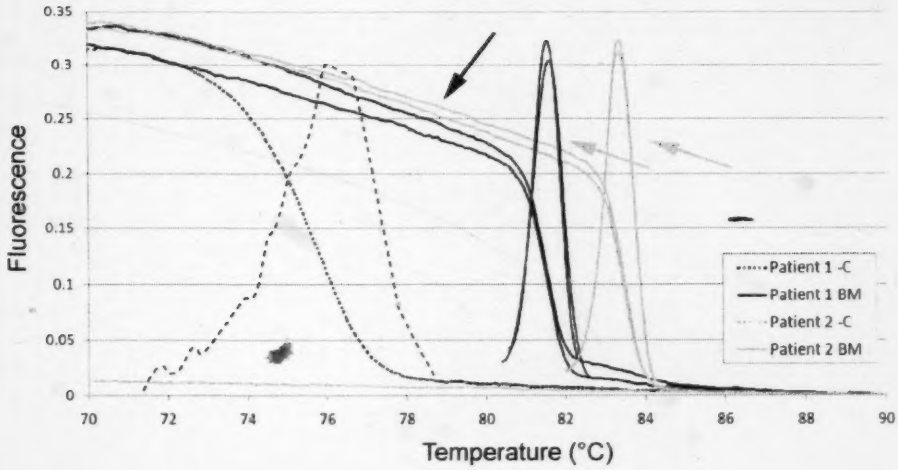


Figure I-4 Melting Curve Using SYBR Green I

The melting curve can only be determined using DNA dyes and verifies the amplification by indicating the amplicon melting properties. Shown are different properties between different patient products (solid lines) and non-specific amplification (dashes lines) from the PCR amplifications in Figure I-2. The lines indicated by the dark arrow show the level of fluorescence over increasing temperature. The temperature at which the specific products melt can be noted as the sudden loss of fluorescence. The lines marked by light arrows show the -dI/dT of the melting curve, the peak indicates the exact melting temperature.

(1) DNA dyes

There are many DNA binding dyes on the market. The best known is ethidium bromide, an intercalating agent used extensively in visualizing DNA in gel electrophoresis under ultraviolet conditions. SYBR Green I (SG) is another DNA binding dye that emits at 520nm, a range more suited for fluorescent detectors. SG is commercially available in quality-tested kits for PCR and available for gel electrophoresis DNA detection. An intercalating agent as well, SG fluorescence is greatly enhanced when bound to the minor groove of double stranded DNA (131). This property of SG can be used to measure the amount of amplification in an RQ-PCR. Fluorescence is measured at the end of the extension phase of a PCR cycle where the amplicon is double

stranded (Figure I-3A). SG has sequence binding specificities and can be influenced by salt concentrations; however, these limitations were negligible for RQ-PCR (131).

As SG fluoresces brightly when bound to double stranded DNA, primer dimer or background amplification can contribute to the fluorescence measured during PCR. However, a melting curve analysis performed after PCR amplification ensures reaction specificity and accurate quantification (Figure I-4). DNA dyes identify the dissociation properties of double stranded DNA denaturing under incremental temperature changes. The amplicon, or any other amplification product, is denatured at a specific temperature dependent on its size and GC content (130). An amplicon will have a discrete and consistent melting profile. In contrast, non-specific amplification products have a broad melting curve and can easily be distinguished from specific products.

Melting curves can be a very powerful tool for identifying single base pair differences. DNA binding imperfections from heterozygous sequence mutations result in heteroduplexed DNA that influences the melting temperature of the amplicon (132). To achieve this high resolution in a melting curve, a DNA dye must be saturating the reaction so that dye being released from one section of the denaturing DNA cannot bind to another section of double stranded DNA. SG cannot be used to the point of saturation due to PCR toxicity (131). Newer dyes like LC Green (Idaho Technologies, Salt Lake City, UT), Eva Green (Biotium Inc, Hayward, CA) and SYTO 9 (Invitrogen Corp., Carlsbad, CA) are used for high resolution melting curves as they can be used to saturation. For situations like RQ-PCR where high-resolution is not necessary, SG is preferred.

SG has been used in RQ-PCR reactions to quantify MRD in acute lymphoblastic leukemia samples (133-135) using the unique Ig rearrangement or T-cell receptor as a clonal marker. It

was also recommended by some that SG not be for used to quantify clonal cells using the IgH locus as 70% of cases contained non-specific amplification (133, 136). In this study (133), only a single patient specific primer was used paired with a consensus primer. This means that one primer was specific to the clonal rearrangement while the other bound to a DNA sequence that is common to all cells. The problem is that the use of even one consensus primer can result in non-target amplification. This amplification will be detected and measured during the RQ-PCR by SG, as found in the studies above. In contrast, both the 5' and the 3' primers can be designed to be specific to the clonal sequence. With this additional measure of precision in primer construction and testing, background amplification will be limited and SG can be used for successful IgH amplification. One study that also used two patient specific primers did not report a problem with background amplification (134). In the MM field, the use of SG in RQ-PCR is rare. In fact, only a single report (a brief correspondence) mentions using SG in MM and simply concludes that usage of SG in RQ-PCR is feasible for use in a Lightcycler (137).

(2) Hydrolysis probes

The second method for RQ-PCR is using hydrolysis probes. This approach, commercially known as TaqMan, utilizes a sequence specific probe linked to a reporter and a quencher fluorochrome (Figure I-3B) (138). The quencher fluorochrome absorbs the fluorescence of the reporter fluorochrome as long as the two are in proximity to each other or while the probe is intact. Upon PCR extension, the Taq polymerase hydrolyzes the probe, separating the quencher fluorophore from the reporter fluorophore. The emissions of the reporter can then be observed, as it is no longer being quenched. The accumulation of the reporter tracks the extent of PCR amplification (139).

The probe location for RQ-PCR in MM is usually placed over one of two types of sequences, patient specific (140) or consensus (140-142). When patient specific probes are used, generally the PCR primers are constructed to bind to consensus sequences that amplify every VDJ rearrangement. The probe only binds to the clonal amplicon, which provides the clonal specificity to be measured during RQ-PCR. This technique can be expensive, as a new specific probe must be generated and labeled with a fluorochrome for each patient. The second common location for RQ-PCR probes are in the conserved regions of the VDJ. The probe will bind to most rearranged sequences based on essential structural areas of the Ig and will not discriminate between the malignant and normal cells. The detection specificity for the clonal sequence comes from primers that are generated to the unique MM rearrangement. While the probe will identify any VDJ sequence, the primers will only amplify the clonal rearrangement. Fully described in Fenk *et al.* (136), the sensitivity and specificity differences between either the patient specific probe with consensus primers or the consensus probe with patient specific primers are equal.

Both of these methods utilize a non-specific element, which has one major complication. The IgH in MM cells have undergone somatic hypermutation, and as a result, mutations may have occurred in these conserved regions where the probe or primers sit. These mutations could lead to a failure of either the consensus primers or consensus probe binding to the target DNA. It was shown in Raab *et al.* that a combination of MM patient specific primers and patient specific probes provided the most sensitive approach compared to either of the above methods for RQ-PCR (143). A strong advantage of using a probe-based system for RQ-PCR is that only the target sequence is measured; non-specific amplification is not detected.

In a study comparing SG to hydrolysis probes in MM, it was found that hydrolysis probes resulted in higher PCR efficiencies, as SG can have some PCR inhibiting properties, and higher sensitivity, as more DNA can be added to the reaction (137). However, quantification of a large numbers of samples can become quite costly using patient specific probes, whereas DNA dye methods avoid the need for expensive probes. Additionally, consensus probes do not detect all gene rearrangements (each VH or JH sequences).

(3) Hybridization probes

The third RQ-PCR technique is using hybridization probes, or more commonly, HybProbes for use in a LightCycler (Figure I-3C) (144). This technique uses two probes that are annealed to the DNA adjacent to each other when the target amplicon is generated. The LightCycler excites the fluorophore on the donor probe. If the donor probe is adjacent to the accepter probe, the photons emitted from the donor fluorophore excite the acceptor fluorophore and its emission is measured. This principle is known as fluorescence resonance energy transfer or FRET; the photon emissions of one fluorophore excite the other. In contrast to SG, the level of hybridization probe fluorescence is detected in the annealing phase of the PCR. As with hydrolysis probes, these probes can be placed in either conserved or specific regions of the Ig locus.

b) Limiting dilution

Another method of quantitative PCR used to determine the level of tumour burden in a laboratory setting is a limiting dilution assay (LDA). Involving no real-time techniques, LDAs use many replicates of serially diluted DNA to quantify the clonal population. The amount of the target sequence is measured by determining the dilution at which the clonal VDJ PCR fails because no target is contained in that dilution reaction. The advantage of LDAs is high

sensitivity. Nested PCRs are used, which are capable of detecting a single copy of the target in high number of background cells. A limiting dilution PCR has a sensitivity down to 10^{-6} while an RQ-PCR has a sensitivity down to 10^{-5} (136). The downside to this method is that it is only semi-quantitative. The percentage is an *estimation* derived by the statistical method of χ^2 minimization or a generalized linear log-log model is that is limited by the number of dilution replicates (145-147). Never the less, Cremer *et al.* determined that LDAs were a more accurate method to measure MRD than qualitative PCR (145). Another downside to LDAs is the requirement for large numbers of PCR replicates. In the study above, 10 PCR reactions were performed for each dilution. The number of reactions required for these assays can be expensive and time consuming. In a comparison between RQ-PCR and LDAs, no significant difference was observed (148).

c) Flow cytometry

The third common method for determining MRD is flow cytometry. Cell surface markers (or CD antigens) create unique expression marker combinations that define populations of cells. In MM, PC enumeration has been used to measure MRD. Normal and often malignant PCs are typically identified by CD38 and CD138 positivity. Additionally, CD56, a marker for NK cells, is expressed on malignant PCs but is low or absent from normal PCs (149) and can be used to phenotype clonal cells. The sensitivity of flow cytometry for detecting MRD is only 0.01% (150). Enumeration of PCs by flow cytometry only measures the PC population, both malignant and non-malignant. However, this disregards any other clonal cell type, as some find reasonable (150). One strong advantage of using flow cytometry is the rapid generation of results. No sequencing of patient VDJ rearrangement or primer generation and testing is required. A panels of CD markers can be used to identify a unique phenotype for malignant PCs in individual patients for quantitation (151). In that study, Sarasquete *et al.* observed a correlation between

flow cytometry enumerated PCs and RQ-PCR (r^2=0.74). Of the samples with an RQ-PCR analysis completed, 25% were negative for flow analysis exemplifying the greater sensitivity of PCR methods.

3. The clinical usage of quantitative MRD in MM

Using qualitative PCR, there was an association with the presence of molecularly defined clonal MM cells and outcome. The problem was that in these studies, only the limited number patients who achieved a clinical CR were eligible for evaluation. In contrast, quantitative PCR can be used for all patients regardless of response. Several studies summarized by Fink *et al.* (136), identify a significant association between the molecularly measured tumour burden and disease stage, and suggested that RQ-PCR could be used to assess drug efficacy. In one study using an LDA (152), the reduction in the tumour burden after high dose therapy indicated two significantly different outcome groups. Patients with decreased BM clonal cells after the first or second round of high dose melphalan therapy had longer event free survivals, while those with higher tumour burden had shorter remissions. This study contained only 20 samples and included only patients who went on to achieve a PR or better after ASCT. In an essential paper by Bakkus *et al.* (153), the levels of clonal cells, as measured by LDAs, were determined in sixty patient samples three months post frontline treatment. No linear correlation was observed between the tumour burden and EFS; however, a cutoff value dichotomizing the cohort into significantly different EFS groups was found. The percent of clonal cells to total cells in a sample was determined and those with a clonal percent below 0.015% had a longer remission than those samples with a higher clonal percent. A cutoff value significantly dichotomizing the patient cohort was confirmed by another group who used a clonal percent of 0.01% and 0.1% (151). In yet another paper, RQ-PCR confirmed that the level of post therapy clonal cells correlates with outcome (154). This paper further observed that a

rise in PB clonal cells could indicate relapse an average of 3 months prior to clinical observations. In a comparison between Taqman and flow cytometry in quantifying the tumour burden from 24 remission BM samples, Sarasquete (151) claimed that both techniques showed the same predictive correlation. A closer look at the data reveals that the association between the PCR results and progression free survival are barely significant with a p-value of 0.04, while for flow cytometry, the p-value was 0.06. This study only used samples from patients who had achieved a CR after treatment, a generally infrequent occurrence.

II. MATERIAL AND METHODS

A. Patients and outcome

Patients in this study were seen at the Cross Cancer Institute between 1995 and 2007. BM aspirates were collected during regular patient visits after informed consent as approved by the University of Alberta Human Ethics Committee. One hundred and thirty nine BM aspirate samples were utilized in the pre-treatment clonal percent study. There were aspirates from 89 previously untreated myelomas and 50 relapsed/refractory myelomas taken immediately prior to a round of new therapy. All the patients here received therapy after the BM aspirate was taken. Patient treatments include single agent or multi agent therapy using combinations of vincristine, doxorubicin, melphalan, prednisone, thalidomide, dexamethasone, cyclophosphamide, LymphoRad, lenalidomide or bortezomib and some received an ASCT. Patient samples were archived as a pellet of cells by preservation at -80°C. The available patient and myeloma characteristics are listed in Table II-1. Remission duration is measured in this

Factor		No. of patient samples/ Total No.*	%
Age	≥65	67/139	48
Albumin	>30g/L	125/135	93
β2m	≥3g/L	85/118	72
Calcium	≥2.5g/L	32/132	24
Creatinine	≥177g/L	22/135	16
Hemoglobin	>100g/L	88/137	64
LDH	>618g/L	19/129	15
t(4;14)	Positive	12/99	12

**Number with factor for group indicated/number known with or without factor for group level*
LDH=Lactate dehydrogenase

Table II-1 Disease and Patient Characteristics for the Cohort Studied Here

study by event-free survival (EFS), defined as the number of days between the BM sampling (at diagnosis or relapse) and clinical relapse (defined by a rise in peripheral or urine M-protein level (85)) or death. Relapse was assessed in non-secretory patients by a rise in serum β2m or increased bone involvement as judged by the treating clinician. Overall survival (OS) is defined as the number of days from diagnosis until last follow-up or death. Alternatively, the number of days between any given time point and death can be measured as time to death (TTD).

Thirty-two remission BM aspirates were also analyzed. These samples were drawn from patients at varying time points during remission (e.g. after ASCT or at CR). EFS is assessed in this cohort. Additionally, time to progression (TTP) is measured as the number of days between the remission BM sampling and relapse/death as above.

B. Cell purification and DNA isolation

BM aspirates and PB samples were processed to obtain mononuclear cells using Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ, USA) as described previously (155). BM mononuclear cell (BMMC) and PB mononuclear cell (PBMC) pellets were archived at -80°C for later DNA isolation. DNAzol Reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the DNA from the archived samples per the manufacturer's instructions. The DNA quantification was performed using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) to ensure equal loading for the PCR applications.

C. Generation of patient specific PCR primers

Primers were generated that were specific to each patient's VDJ rearrangement as previously described (156). Briefly, a panel of 12 V region 5' primers are used with either an IgA or IgG constant region 3' primer to PCR amplify any rearranged Ig in the BMMCs transcriptome. RT-PCR reactions that result in appropriately sized band in the agarose gel electrophoresis were

submitted for direct DNA sequencing. If clear, monoclonal sequences were derived from the direct PCR product, primers were designed over the CDR1 or 2 region and the CDR3 region. To find areas containing unique sequences to generate primers for, the derived monoclonal sequence is compared to an unmutated VDJ region using online algorithms from http://imgt.cines.fr/IMGT_vquest/vquest?livret=0&Option=humanIg. The primers were made and tested on the same patient's bulk BMMC sample. In parallel with a standard water PCR control, samples from other patients with similar VH regions are used to control for promiscuity of primers. When possible, primers were further validated using single sorted $CD38^{+}CD138^{+}$ (and when possible, $CD56^{+}$) PCs from the same patients BMMC population. Twenty-four single PCs are tested for PCR positivity using the patient specific primers; a success rate of 75% or higher is desired.

D. Limited dilution assay

LDAs were run to verify the RQ-PCR results. This assay used nested PCR with patient specific primers and control gene (β2m) primers. A nested PCR reaction is a two-stage PCR reaction where product from the first stage is used as template for the second. Primers for the second stage reaction must sit internal of those in the first stage. A dilution series was made from the patient's DNA using sterile water; five PCR reactions were performed for each dilution. In stage one of the PCR, 0.4μM of each primer, FR1c (AGGTGCAGCTG(GC)(AT)G(GC)AGTC(GAT)GG), JHc (ACCTGAGGAGACGGTGACC(AG)(GT)(GT)GT), B2m 3'-2 intron (TCAGATGGGATGGGACTCA) and B2m 5'(CCAGCAGAGAATGGAAAGTC) were added to DNA, 2.5μL 10x PCR Buffer, 0.02mM dNTPs, 2μM $MgCl_2$, 1U recombinant *Taq* and water to 25μL (the 10X PCR Buffer, $MgCl_2$ and *Taq* were from Invitrogen, Carlsbad, CA, USA). The amplification conditions were: 95°C for 2min; 28 cycles of 94°C for 30sec, 60°C for 30sec and 72°C for 1min; followed by 72°C for 5min. The second stage PCR was split into separate

reactions, one with patient specific VDJ primers and the other with β2m primers. The VDJ reaction contained 0.4μM of each primer (NIR5' (TTTTGATACTGACGATGAGGAAG) and NIR3' (GGAGTGAACTGCTATCGATGAC)) added to 2μL of the stage one PCR reaction with the remaining reagents as indicated above. The β2m reaction differed only in the primers used (B2m 3'-2 and B2m 5'int (TGTCTTTCAGCAAGGACTGG)) and that only 1μL of the first stage reaction was added as template. The second stage PCR cycling conditions were: 95°C for 2min; 35 cycles of 94°C for 30sec, 60°C for 30sec and 72°C for 30sec; and 72°C for 5min. The second stage PCR was subjected to size separation by 2% agarose gel electrophoresis. A reaction was deemed successful if the appropriate β2m band was visible. The matched reactions were counted as positive for clonal DNA if the patient specific PCR generated the appropriately sized band. For each dilution, the number of cell equivalents was calculated utilizing the formula $7.11\mu g=1e^{6}$ cells. The percent of clonal cells was calculated using the generalized linear log-log model from the online limiting dilution analysis program from http://bioinf.wehi.edu.au/software/limdil/index.html. This program estimates the clonal percent when supplied with cell equivalents added, total reactions performed for each dilution and positive clonal reactions for each dilution. The numbers generated must be taken to the power of -1 to calculate the percent fraction (e.g. 12.5^{-1} *(or 1/12.5)* * 100 = 8%).

E. Clonal cell enumeration by RQ-PCR

RQ-PCR was performed on genomic DNA from BMMCs and PBMCs using the DNA Engine Opticon 2 (Bio-Rad Laboratories, Hercules, CA, USA). The DyNAmo HS SYBR Green qPCR Kit (Finnzymes, Espoo, Finland) was used for the RQ-PCR reaction as follows: 10 μl of DynAmo Master Mix, 0.25μM of each patient specific primer, genomic DNA and water to 20μl. For the patient specific reactions using BM samples, 150ng of DNA was added, and 200ng of DNA was added to the PB reactions. The control PCR amplified an intron/exon boundary of the β2m gene

(to eliminate amplification by cDNA) and 75ng or 100ng for each BM and PB reactions, respectively, of genomic DNA was added to the PCR to compensate for the two genomic copies of β2m versus the single clonal VDJ copy. The β2m primers are B2m5'int2, TTGTTGGGAAGGTGGAAGCTCAT, and B2m3'exo2, ACCCAGACACATAGCAATTCAG. The cycling conditions for all the RQ-PCR reactions were 15min at 95°C, 45 cycles of 15sec at 94°C, 30sec at 60°C and 30sec at 72°C, and a final extension phase of 2min at 72°C. Opticon Monitor 3 software (Bio-Rad Laboratories, Hercules, CA, USA) was used to analyze the RQ-PCR data. The threshold was placed at the midway point of the log amplification curve to generate the C(T) value for each RQ-PCR reaction.

SG further analyzes the amplicon through melting curve analysis. Here, the melting curve was generated by measuring the level of fluorescence in 0.1°C increments for 1sec each while increasing the temperature from 70°C to 90°C. The Opticon Monitor 3 software calculates a –dI/dT to determine the melting point of the amplicon. The melting temperature (Tm) confirms the correct amplicon has been amplified, and identifies any amplification impurity, e.g. primer dimer, background amplification or additional products. Specific target amplification results in a very clean melting profile with a single –dI/dT peak that is typically in the range of 1 degree in width. The height of the –dI/dT peak is proportional to final concentration of the amplicon. Prediction of the Tm is usually not performed as only very simple algorithms to determine Tm have been developed. One such program is found at www.bioinfo.rpi.edu/applications/hybrid/twostate-fold.php. However, using the sequence for the β2m reaction the predicted Tm is 81.6°C while the actual Tm is between 78.8°C and 79°C. The true nature of melting DNA is unpredictable as changes in salt and amplicon concentrations alter melting (157).

A cloned amplicon was used to generate a standard curve to determine the exact molecule count for each reaction. The β2m and patient specific IgH VDJ amplicons were cloned using pGEM-T Easy Vector System I (Promega, Madison, WI, USA) and Subcloning Efficiency DH5α cells (Invitrogen, Carlsbad, CA, USA) per manufacturer's instructions. Plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen Inc., Mississauga, Ontario, Canada) and the identity of the insert was confirmed by PCR with the primers used to generate the original amplicon. A 10-fold dilution series was constructed and at least three replicates for each dilution were subjected to RQ-PCR analysis concurrent with the BMMC sample. The software generated a standard curve where the slope defines the relationship between the C(T) value to a molecule count. Cloned amplicons, for the purpose of a standard curve, were generated for only the first 53 patients from the cohort. After testing, an aggregate curve was used in place of a patient specific curve. Discussed in more detail below, the aggregate curve was generated from a regression line using the series of individual standard curves, eliminating the excessive time needed to clone each patient's amplicon. The percentage of MM clonal cells, termed VDJ%, was calculated based on the molecule count of patient specific VDJ targets to the β2m molecule count. The resulting percentage represents the number of clonal cells present in the total mononuclear population. Where both a pre-treatment and remission VDJ% were available, the times reduction was calculated by dividing the pre-treatment VDJ% by the remission VDJ%.

F. Determination of BM PC percent

Flow cytometry was used to determine the level of PCs in the BMMC populations using cell staining data collected at the time the original samples were processed. No further cytometry analysis was performed for this thesis. Flow cytometry data files from approximately the last 10 years of BM aspirates were re-analyzed. Forty-seven patient samples were used and

new gating parameters were applied to maintain consistency between each sample. Due to the length of time over which samples had been collected, several different sets of staining markers were used: 25 samples were gated for $CD138^+38^+56^+$ cells, 4 were $CD138^+38^+56^-$, 14 were $CD138^+38^+$ and 4 were $CD38^+$. Each sample was analyzed for the markers in an ungated background to include all events counted by the detectors, and after setting gates to include only the live cell events (Gate D in Figure II-1A). The PC% is defined as the number of events in the PC marker gates in an ungated background or using the live cell gates. A representative result of PC% determination for $CD138^+38^+56^+$ cells is shown in Figure II-1. Additionally, when available, clonotypic PCR of single sorted PCs gave further information of the number of PCs that are clonal, as described in section II.C. If, for example, only 20 of 24 PCs were clonal by PCR then the final PC% would be 83% of the PC% determined from flow cytometry analysis.

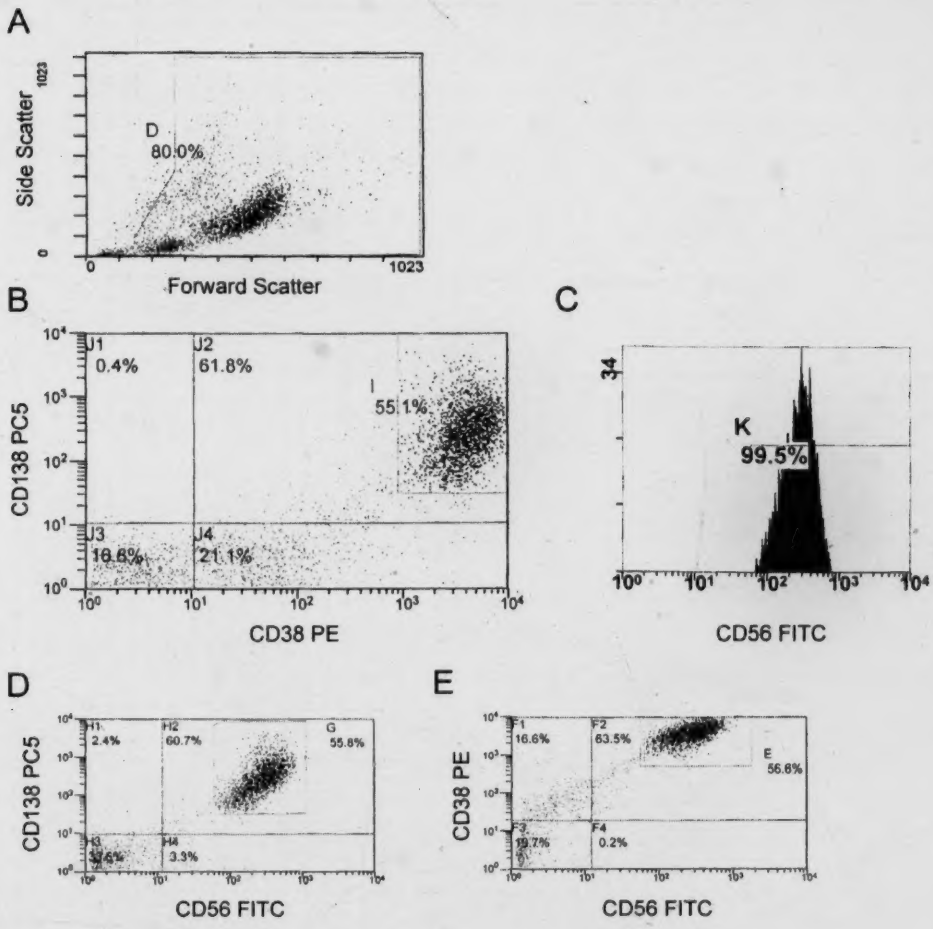


Figure II-1 Representative BM Flow Analysis for Determining the PC%

Forward and side scatter events indicate where live cells are found (gate D) to exclude any dying or dead cells and particulate matter (A). Gate I is placed around the $CD138^{+}CD38^{+}$, gated on D, events (B). Plot C indicates the number of I and D events that are CD56+, K (C). K identifies the number of triple stained events by gating the I events from plot B against the CD56 gate. The live-gated PC% is K gated (I and D) and the ungated PC% is K ungated (I alone). Plots D and E confirm the triple positive population of PCs. The darkest events in each plot represent the K events.

G. Statistical tests

To test for correlations with outcome, including event free survival and overall survival, Kaplan-Meier curves were generated using GraphPad Prism 5 and a logrank statistic generated a p-value. Correlation tests were performed using Pearson's Correlation, Spearman's Correlation or Fisher Exact tests wherever applicable. A Pearson's correlation is a parametric test that treats the variables as continuous. Continuous values are defined as those that do not fall under discrete categories but exist in a range of numbers, like the percent of PCs. In a Pearson's correlation, each data point is taken as the numeric value. In contrast, Spearman's correlation is a non-parametric analysis that places the continuous variables in order and tests their *ranks* in relation to each other as opposed to their actual values. Parametric tests assume the population fits normal distribution as compared to non-parametric tests, which do not. The Fisher Exact test requires two dichotomized data sets and looks at statistical connection of one to the other. This type of data is known as categorical, for example: above or below the median or presence of a translocation. A Kruskal-Wallis test or an ANOVA was used to compare the medians of more than two groups of data. These tests were done using Sigma Plot Software. A maximum likelihood analysis was done on the VDJ% and other clinical variables by Ms. S. Ghosh of the Cross Cancer Institute.

III. RESULTS

It has been well documented that levels of minimal residual disease during remissions of hematological cancers are significantly associated with event free survival. However, no work has previously been done to identify if the disease burden prior to treatment is correlated with outcome. The work discussed here involves mostly molecular techniques, specifically SG RQ-PCR, to measure tumour burden in the bone marrow and blood of patients with multiple myeloma.

A. SYBR Green I sensitivity in the RQ-PCR assay

It is important when utilizing a new technique, like RQ-PCR using SG in MM, that the lowest level of positive detection be resolved to understand the limitations of the method. Occasionally, an RQ-PCR does not amplify any target, a negative result. After the DNA quality has been checked, by amplification of the β2m gene, and the VDJ primer accuracy has been checked, by amplification of a positive control, a sample negative for VDJ amplification can be deemed below the level of detection. For calculations, a value must be given to these samples in order that they may be included in the statistics. To this end, the detection threshold for the RQ-PCR SG system must be determined. The Opticon DNA Engine quantitative PCR machine and the SG kit were used to determine the lowest level of detection. DNA from a MM PC cell line (LP-1) with a known clonotypic IgH VDJ rearrangement was diluted in a constant amount of normal PBMC DNA to simulate patient samples with different clonal percentages. DNA was purified from normal PBMCs and spiked with cell line DNA. The x-axis of Figure III-1 illustrates the dilution series generated. The LP-1 VDJ reactions were performed here using the LP-1(LC) 5'-3 (5' TACCAACAGACGCCAGGCC 3') and LP-1(LC) 3'-3 (5' CAGTAATAGTCGGCCTCATCCC 3') primers. To each VDJ reaction, 150ng of DNA from each dilution was added, and to each β2m

reaction, 75ng of DNA was added to compensate for two β2m gene loci compared to the single rearranged VDJ locus. The average molecule count for each dilution, determined using a standard curve for the VDJ and β2m PCRs, was used to calculate the percent of clonal cells present (Figure III-1). Typically, a standard curve is required for each different amplicon, one for the β2m PCR and one for each patient VDJ PCR, to compensate for varying PCR amplification efficiencies. However, an aggregate standard curve was generated using the standard curves of several VDJ amplicons for use with all VDJ PCRs. Further explanation and verification of the aggregate standard curve is discussed below (III.E). The lowest dilution with detectable VDJ signal was 0.001%, 2 of 3 samples were positive, which is equivalent to 1:100 000 cells. Patient samples that fell below this threshold value were arbitrarily assigned a value of 0.0001% for inclusion in the analysis.

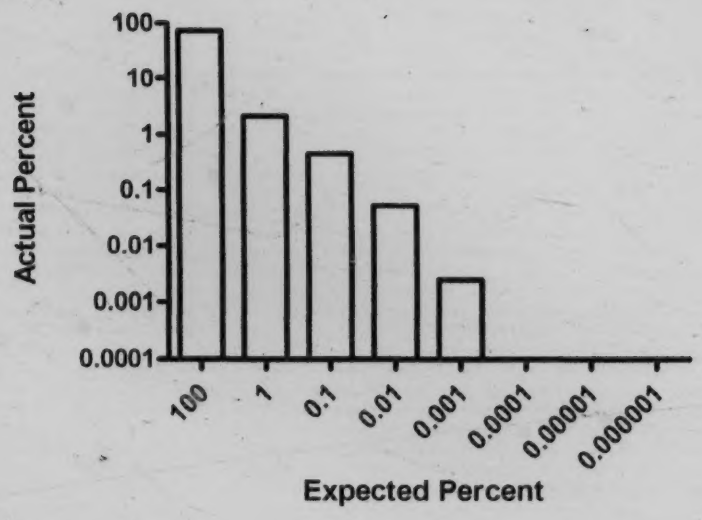


Figure III-1: SYBR Green I Sensitivity

RQ-PCR was performed on 150ng of LP-1 cell line DNA diluted to known percentages in normal PBMC DNA in order to determine the lowest level of detection for the SG system. The bars represent the mean of 3 replicate PCR reactions to determine the VDJ% for each dilution noted by comparing the VDJ PCR molecule count plotted from the VDJ control curve (using the aggregate curve, section III.E) to the β2m molecule count using half the DNA. The absence of bars at the 0.0001%, 0.00001% and 0.000001% levels indicate the absence of amplification.

B. Limiting dilution assay

SG RQ-PCR has frequently been utilized in experiments in other hematological malignancies. It is important that SG RQ-PCR for use in MM be confirmed for accuracy using another quantification technique commonly used for clonal enumeration in MM. LDA has been used for years prior to real-time techniques to estimate the levels of clonal cells in a sample. Here, LDAs were used to confirm the results from two SG RQ-PCRs tests. Figure III-2 shows an LDA from a BM sample (Figure III-2A) and PB sample (Figure III-2B). The VDJ% was calculated using the cell equivalents added and the PCR results for each dilution using a complex equation from an web-based program http://bioinf.wehi.edu.au/software/limdil/index.html). For the two BM and PB samples, the clonal percent estimate based on an LDA and based on SG RQ-PCR are:

	LDA Estimate	SYBR Green RQ-PCR
BM	7.97%	7.93%
PB	0.0194%	0.0156%

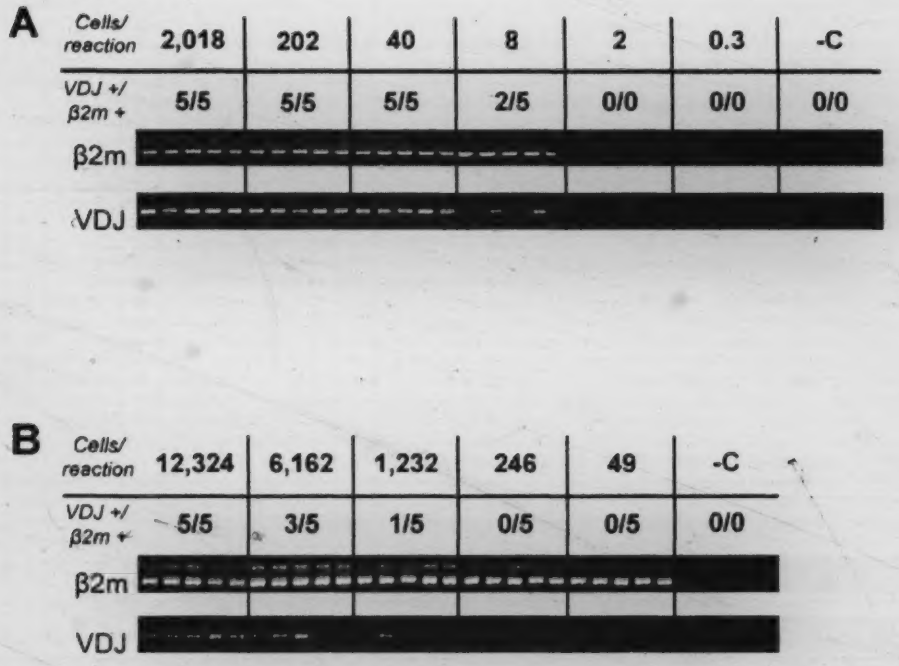


Figure III-2 Limiting Dilution Assay PCR Results

DNA from a BM sample (A) and a PB sample (B) was added to a nested PCR reaction. The cell equivalents added are noted at the top of each figure. Shown are five replicates of each dilution. The β2m reactions in the upper panels indicate the number of successful reactions and the patient specific VDJ PCR in the lower panels indicate those reactions containing clonal target.

C. Pre-treatment VDJ% correlates with EFS but not OS

One hundred and thirty nine BM aspirate samples from MM patients were collected shortly prior to treatment, of which, 89 were from previously untreated MMs and 50 were relapsed/refractory MMs starting a new line of therapy. Samples were collected from patients treated with a wide variety of regimens and enriched for BMMCs. RQ-PCR was performed using the DNA from these pre-treatment BM samples and an aggregate standard curve (further discussed in section III.E) was used to determine molecule count for VDJ targets and β2m targets. A percent of clonal cells in the BMMC population, termed VDJ% can then be determined. Based on the VDJ%, the patient cohort was dichotomized into two groups: above or below the median, 18.2%. The event free survival (EFS), was significantly different between these two groups using a Kaplan-Meier analysis (p=0.0269) where those patient samples with a VDJ% falling below the median had a longer EFS (Figure III-3A). The hazard ratio (HR) is the relative risk between two data groups and represents the difference in the survival curves. The HR between patients below the median VDJ% to patients above the median VDJ% is 0.6259 (95% confidence 0.4010 to 0.9772). The median EFS time for above and below the median VDJ% was 490 days and 961 days, respectively.

Sub-dividing the patient cohort to include only samples from patients not treated with an ASCT revealed a continued significant association between VDJ% and EFS in a Kaplan-Meier analysis (p=0.0081, HR=0.4870, 95% confidence 0.2858 to 0.8296) (Figure III-3B). In contrast, the VDJ% from the remaining patient samples, those who received an ASCT as part of their treatment regimen, did not show a significant association with EFS (p=0.7612, HR=0.8824, 95% confidence 0.3566 to 2.126) using Kaplan-Meier curve analysis (Figure III-3C). Significantly different EFS categories were observed between patients receiving a stem cell transplant (1012

days), non-ASCT treated patients with a VDJ% below median (740 days) and non-ASCT treated patients with a VDJ% above the median (319 days) (Figure III-3D and E).

Of note, 27 samples were from patients to whom a previous sample has been included in the cohort. Twenty patients have a relapsed and untreated sample in the cohort while 7 have two relapses included. Different samples were drawn from the patient prior to different treatment cycles. Including only those samples that were collected the earliest in a Kaplan-Meier curve continued to show a significant association between VDJ% with EFS ($p=0.0233$, $HR=0.5529$).

The VDJ% prior to therapy was significantly associated with EFS, but the VDJ% from samples taken at diagnosis were not associated with overall survival (OS) (Figure III-4A). OS is defined as the number of days from the collection of the diagnosing BM aspirate until death. The data above shows that the VDJ% from ASCT treated patient samples did not correlate with EFS, while samples from patients not receiving an ASCT did correlate. Thus, removing the ASCT treated patients from the OS analysis may reveal a significant association between VDJ% and OS; however, the data identified no significant association (Figure III-4C). The *total* length of disease is measured by OS, from diagnosis to death and many samples were not included as the BM aspirate was collected during relapse. However, it is possible to utilize all the samples by measuring the number of days from the BM aspirate to time to death (TTD). As with OS, the VDJ% from the pre-treatment BM samples did not correlate with TTD (Figure III-4C).

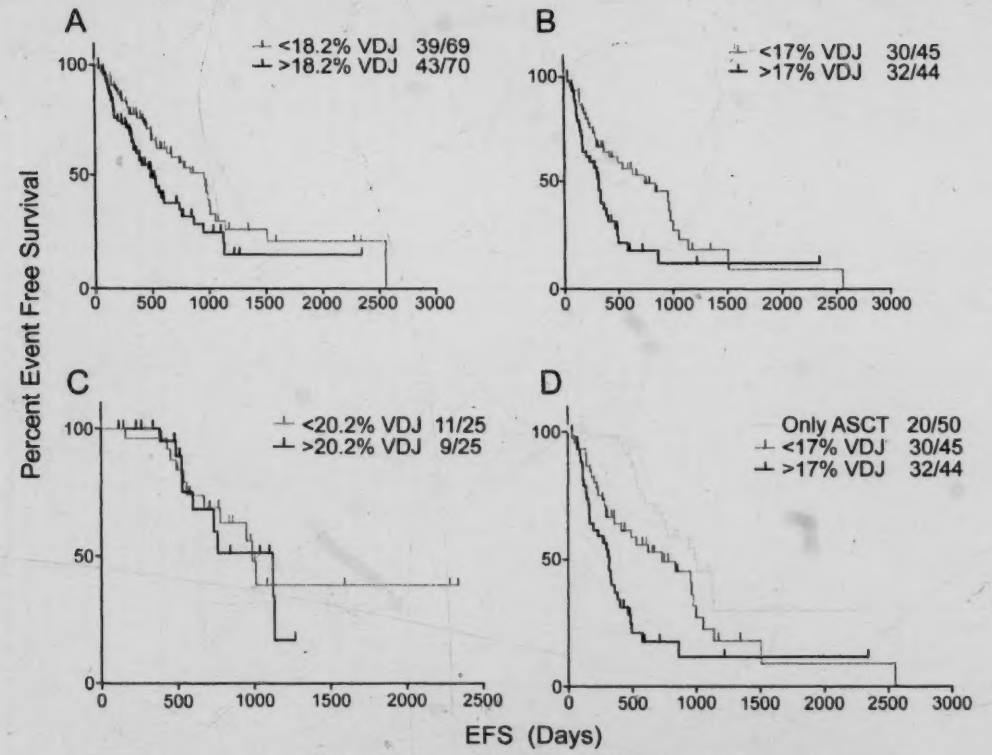
This cohort of patient samples was analyzed using maximum likelihood tests for associations between other factors and EFS or OS as continuous or dichotomous variables (Table III-1). All these factors have previously been shown to associate with outcome, whether EFS or OS. With a p-value of <0.05 indicating significance, only a select few variables from this study's cohort

show significant correlations with outcome. The pre-treatment VDJ%, when treated as a continuous variable, was not associated with EFS. OS was not predicted when VDJ% was treated as a continuous or a dichotomous variable. A significant association was found between pre-treatment VDJ% and β2m, LDH, hemoglobin or creatinine, but not with any other factors noted in Table III-1.

	EFS*		OS**	
	Continuous	Dichotomous	Continuous	Dichotomous
Pre-treatment VDJ%	0.1961	**0.0285**	0.8067	0.2139
Age	0.319	0.3047	0.1867	0.2096
Albumin	0.7562	0.122	0.7054	0.1604
Calcium	0.5761	0.4775	0.7553	0.2967
Hemoglobin	0.2886	0.2657	0.8441	0.2692
LDH	**0.0001**	**0.0127**	**0.0423**	**0.0041**
Creatinine	0.3722	0.8067	**0.0287**	0.3291
β2m	**<.0001**	0.3086	**<.0001**	0.0739
Gender	*n/a*	0.2752	*n/a*	**0.0485**
t(4;14) Status	*n/a*	0.1158	*n/a*	0.622
VH3	*n/a*	0.2116	*n/a*	0.2542

**EFS=Event Free Survival **OS=Overall Survival*
LDH=Lactate dehydrogenase

Table III-1 Correlations of Patient and Tumour Characteristics to Outcome Using Maximum Likelihood Estimates



E

	Logrank p-value	Hazard Ratio	Median EFS: upper half	Median EFS: lower half
All Samples (A)	0.0269	0.6259	490	961
Non-ASCT Samples (B, D)	0.0081	0.487	319	740
ASCT Samples (C)	0.7612	0.8824	763	1012
Non-ASCT Above Median (D)	<0.0001	0.2361	319	1012
Non-ASCT Below Median (D)	0.0149	0.5075	740	1012

Figure III-3 Kaplan-Meier Analysis of Pre-Treatment BM VDJ% and EFS

The VDJ%, dichotomized by the median, shows a significant correlation with EFS (A) where those who fall below the median (light line) have longer EFS than those who fall above the median (dark line). There were 39 relapses out of 69 patient samples below the median and 43 of 70 above the median. When patient samples were separated to include only those not receiving an ASCT the significance remains (B). When the remaining patients, those receiving an ASCT, were analyzed, the significant correlation was lost (C). D represents the Kaplan-Meier curve of three significantly different EFS categories: lightest line=all ASCT treated patients, medium grey line=patients not treated with an ASCT who fall below the VDJ% median and the dark line=patients not treated with an ASCT who fall above the VDJ% median. E summarizes the logrank p-value, hazard ratio and median survivals for the graphs above.

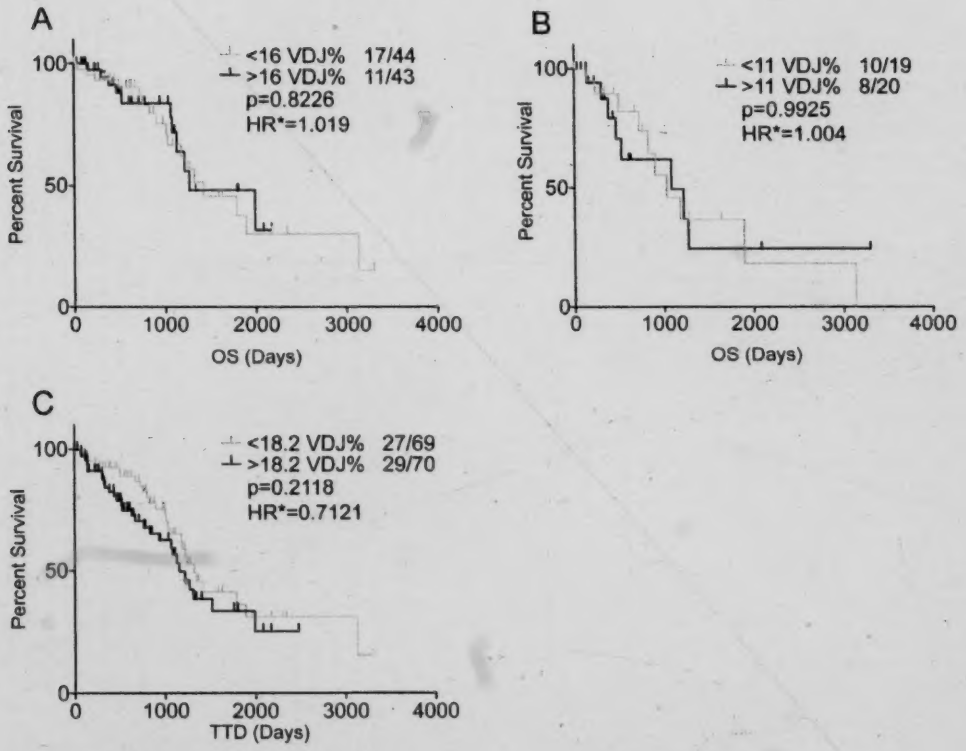


Figure III-4 Kaplan-Meier Curves of Pre-Treatment BM VDJ% and OS or TTP

The VDJ% from BM samples taken at diagnosis, dichotomized by the median, did not correlate with OS (A). There were 17 deaths out of 44 total patients below the median (light line) and 11 out of 43 above the median (dark line). If the patients who received an ASCT were removed, the survivals remain not significant (B). If all the patient samples were included and survival was measured as the time the BM sample was taken to death (TTD) the logrank statistics show no significant correlation (C).

*HR=hazard ratio

D. The VDJ% from remission BMs did not associate with outcome

The remission BM VDJ% has been significantly associated with outcome in other labs. These studies used samples from previously untreated patients who received an ASCT as part of their front-line therapy. Little or no research has attempted to understand the clonal percent for patients in remission after treatment with new therapies such as bortezomib or lenalidomide. Nor has the clonal percent from remission samples after treatment for *relapsed* MM been studied.

The tumor burden in 32 remission/post-treatment BM samples was quantified using SG RQ-PCR. The samples were from different patients with different treatment courses, including frontline VAD/ASCT and bortezomib/ASCT, or relapse samples treated with Dex, bortezomib or lenalidomide. Some remission samples did not have detectable VDJ% values. As mentioned in section III.A, the samples that are negative by patient specific PCR were assigned the value of 0.0001% as their VDJ%.

In a Kaplan-Meier analysis, the remission VDJ% did not show a significant correlation with EFS (p=0.6934, HR=0.8026) (Figure III-5A and Table III-2). Of the 25 patients with matched pre-treatment and remission samples, the relative reduction of VDJ% was calculated. This 'times reduction' was not associated with EFS in a Kaplan-Meier analysis (p=0.4620, HR=0.6041) (Figure III-5B and Table III-2).

As the remission BM samples were taken at varying times in the treatment course, an alternative measure of outcome is time to progression (TTP), which was calculated as the number of days between the remission BM aspirate extraction and the time of clinical relapse.

Both the remission VDJ% and times reduction were not significantly associated with TTP (Figure III-5C and D and Table III-2).

The remission VDJ% values and times reduction from patients treated with either VAD or bortezomib as frontline therapies are seen in Figure III-6A and B. Of the patients treated with VAD, all the remission samples were taken after high-dose therapy and ASCT. In the bortezomib treated patient remission samples, 3 were taken after ASCT and 2 before. In an unpaired t-test, the mean remission VDJ% and times reduction are not significantly different between the two groups ($p=0.6425$ and 0.057, respectively). In remission samples from patients treated for relapsed MM with Dex, bortezomib or lenalidomide, the VDJ% is noted in Figure III-6C. A one-way ANOVA, which is a parametric test that compares the means of more than two sets of data, indicated no significant difference between the groups ($p=0.5241$). However, in a non-parametric examination, a Kruskal-Wallis test, the means were significantly different ($p=0.0428$). The times reduction data for this group is insufficient for statistical examination. These data indicate no significant difference in the remission VDJ% or time reduction in patients treated with standard therapies compared to those treated with newer, better-tolerated therapies.

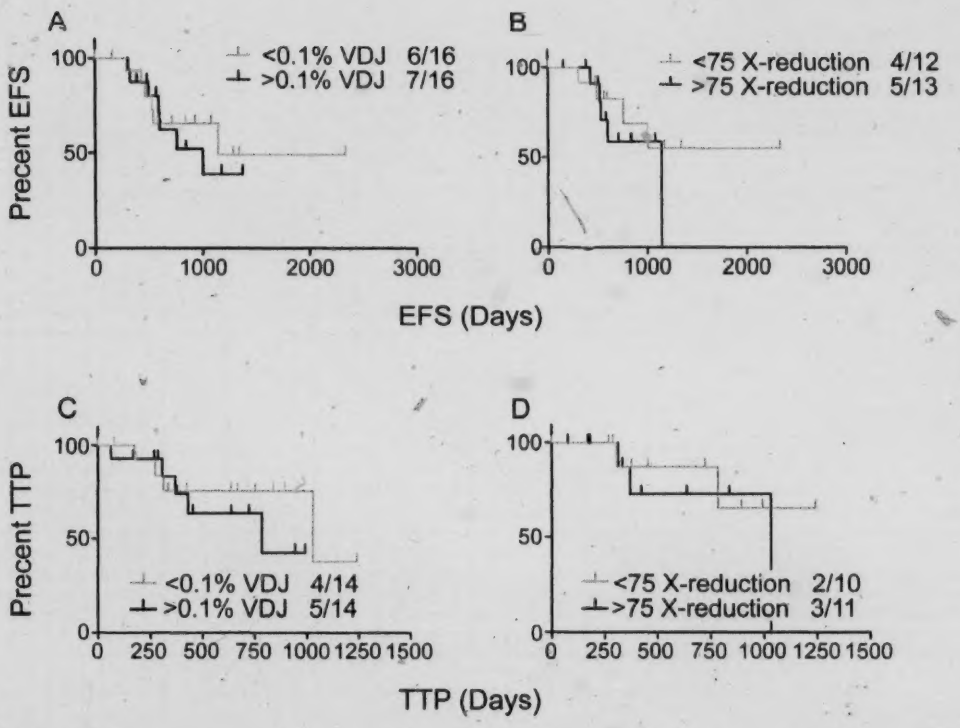


Figure III-5 Kaplan-Meier Curves of Remission BM VDJ% and Times Reduction of the Tumour Burden

The remission BM VDJ% does not correlate with EFS (A). Six relapses were observed in 16 patients for samples below the median VDJ% and 7 relapses occurred in 16 patients with VDJ%s above the median. The times reduction of the pre-treatment VDJ% to the post treatment VDJ% did not correlation with EFS (B). The time to progression (TTP), as measured from the time the remission BM sample was taken to the point of relapse, was not predicted by VDJ% (C) or times reduction (D).

Logrank Test	*p-value=	HR*
Remission VDJ% vs. EFS	0.6934	0.8026
Times Reduction vs. EFS	0.4620	0.6041
Remission VDJ% vs. TTP	0.4407	0.5792
Times Reduction vs. TTP	0.5430	0.5765

Table III-2 Logrank statistics of Figure II-5

**HR=hazard ratio*

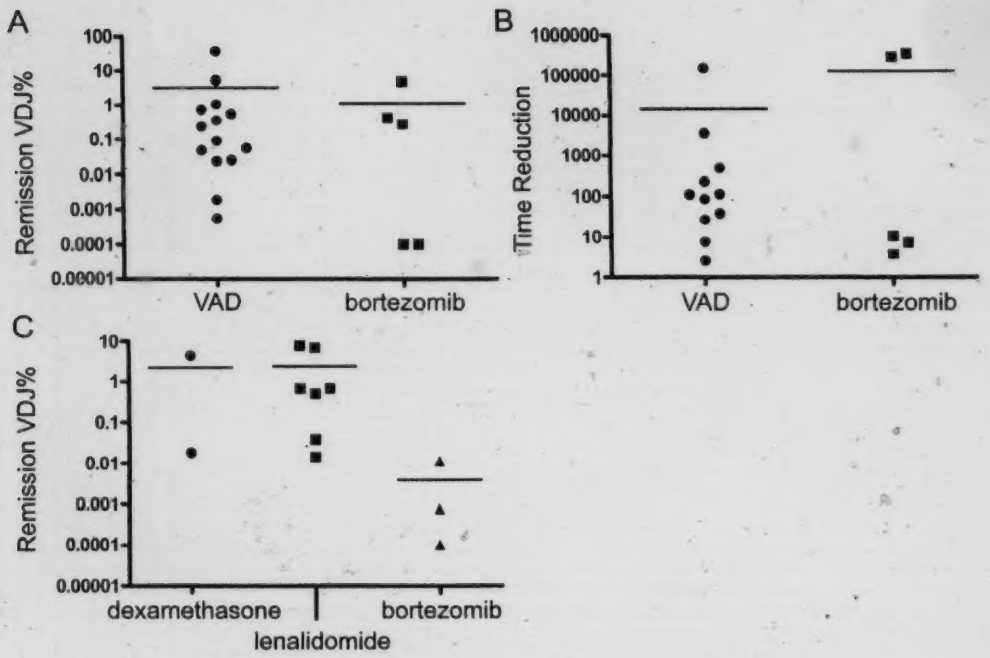


Figure III-6 Remission VDJ% Levels and Times Reductions of Remission BM samples.

The post treatment VDJ%s from a front-line VAD and front-line bortezomib just prior to ASCT (A). The times reduction of the VDJ% in the same samples are plotted in B. In relapsed patients, the VDJ% is noted in C after having been treated with dexamethasone, lenalidomide or bortezomib. The line through each group represents the mean. Dex=dexamethasone

E. Aggregate curve

To utilize the truly quantitative nature of RQ-PCR a standard curve must be generated for each amplicon to ensure the measurements account for the specific PCR efficiency for each primer set. Individual standard curves were generated using plasmids containing a specific clonotypic IgH VDJ from the first fifty-three patients with at least three PCR replicates of at least three dilution points. The VDJ% values from pre-treatment BM samples were generated using individual standard curves and a significant association with EFS was observed (Figure III-7C light lines). When the individual standard curves were studied, it was observed that the slope of each curve was very similar among the patients. To increase throughput, the use of a single aggregate standard curve was investigated for all the VDJ RQ-PCR reactions. The C(T) values generated from the individual standard curve reactions were plotted together to form an aggregate regression curve (Figure III-7A). The resolved slope formula (Equation 1) was used to generate new aggregate VDJ% values using the original samples C(T) values where x was the C(T) value and y was the molecule count.

Equation 1: $$y=10^{(-0.2774x+9.708)}$$

A significant correlation between the VDJ% values calculated with the individual standard curves versus those using the aggregate standard curve (Figure III-7B) was found using a Spearman's correlation ($p<0.0001$, $r=0.5530$) and a Fishers Exact test based on the medians ($p<0.0001$). The VDJ% values calculated using the two methods were plotted in a Kaplan-Meier curve and a logrank test showed that both generate significant associations with EFS (Figure III-7C). Only six of forty-four samples moved from below the median to above and vice versa resulting in an agreement of 86%. All further samples were then evaluated using the aggregate VDJ standard curve.

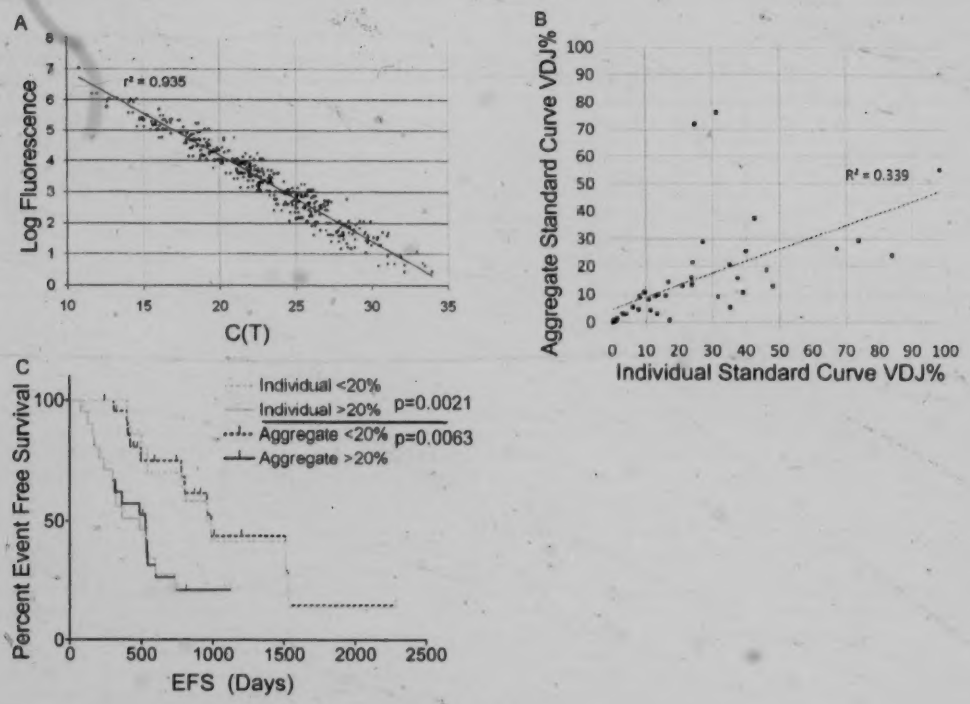


Figure III-7 Generation and Validation of the Aggregate Standard Curve

(A) The C(T) values of dilution curves from 53 cloned IgH VDJ amplicons were plotted against the relative log fluorescence and a best-fit line generated the aggregate standard curve formula. At least three 10-fold dilutions with at least three replicates were used per cloned amplicon. (B) VDJ% values derived from individual standard curves plotted against the same C(T) values analyzed with the aggregate standard curve generated in (A). A Kaplan-Meier curve (C) compares the EFS in patients using the individual or aggregate standard curves. The light solid and hatched lines represent the VDJ% values derived from the individual standard curves above and below the median, respectively. The dark solid and hatched lines represent the VDJ% values derived from the aggregate standard curve above and below the median, respectively. The p-values were generated using the logrank test.

F. BMPC does not significantly associate with EFS.

Part of the clinical evaluation of MM is to measure the percentage of PC in the BM aspirate, termed a BM plasmacytosis (BMPC). Traditionally this measure is not associated with outcome. An investigation into the relationship between this clinical measure and the VDJ% revealed a significant but weak correlation between the pre-treatment VDJ% and the sample-matched BMPC percent using both a Pearson and Spearman correlation (Figure III-8A and Table III-3). The two measures were also significantly correlated in a Fisher Exact Test where the median served to dichotomize the patient samples for both data sets (p=0.0008). In a Kaplan-Meier analysis, where the median dichotomized the group, the BMPC was not significantly associated with EFS (Figure III-9A). As not all the samples had a matching BMPC reported, it is important to verify that the pre-treatment VDJ% from this smaller cohort of patient samples is still able to predict outcome. When the VDJ% dichotomized this same sub-cohort for a Kaplan-Meier analysis, a significant correlation was observed (Figure III-9B).

As noted in section III.C, the exclusion of ASCT treated patients from a Kaplan-Meier analysis comparing EFS to VDJ% decreased the p-value and resulted in a more significant association. If the ASCT treated patients were excluded from the Kaplan-Meier analysis of BMPC and EFS, there continued to be no significant association (Figure III-9C). Alternatively, when the sample matched VDJ% values from this cohort were analyzed, there again was a significant association with EFS (Figure III-9D).

The remission BM samples also had a BMPC performed and these numbers can be compared against the remission VDJ% values (Figure III-8B) and the times reduction (Figure III-8C). Using Pearson's correlation, neither the remission VDJ% nor the times reduction had a significant correlation to BMPC (Table III-3). However, in a Spearman's correlation, both the

remission VDJ% and the times reduction were significantly associated with the remission BMPC, while very weakly (Table III-3).

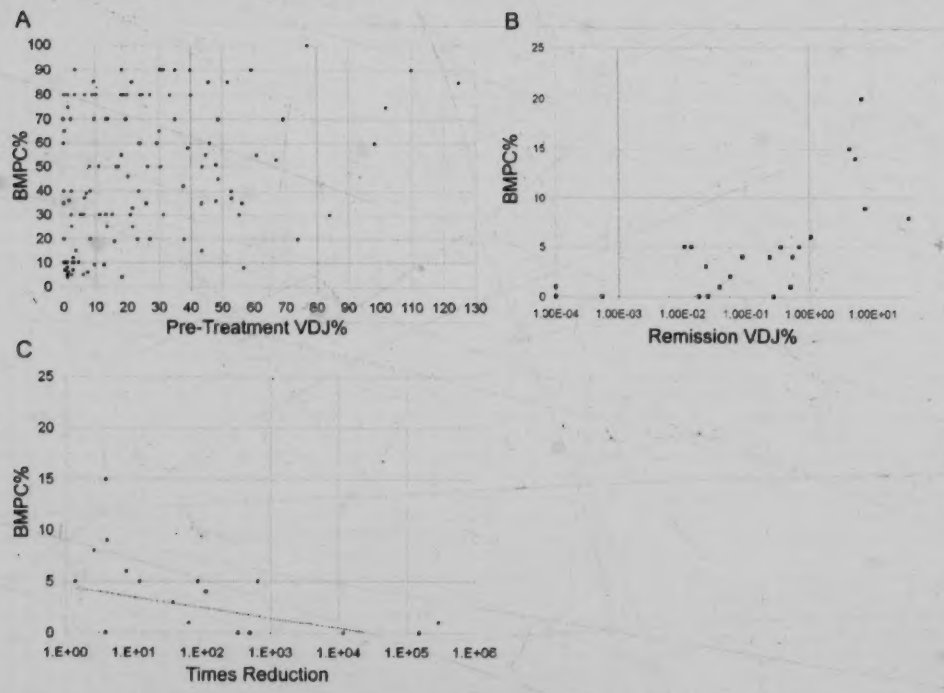


Figure III-8 Association Between the BMPC and the VDJ%

Pre-treatment BMPC percents compared to pre-treatment VDJ% values (A). Remission BMPC percents compared to remission VDJ% (B) and times reduction (C).

Table III-3 Correlation Statistics of Figure III-8

Matched BMPC vs.	Spearman's (p-value)	Person's (p-value)	r^2
Pre-treatment VDJ%	**< 0.0001**	**0.0002**	0.1068
Remission VDJ%	**< 0.0001**	0.0707	0.1409
Times Reduction	**0.0084**	0.2345	0.0871

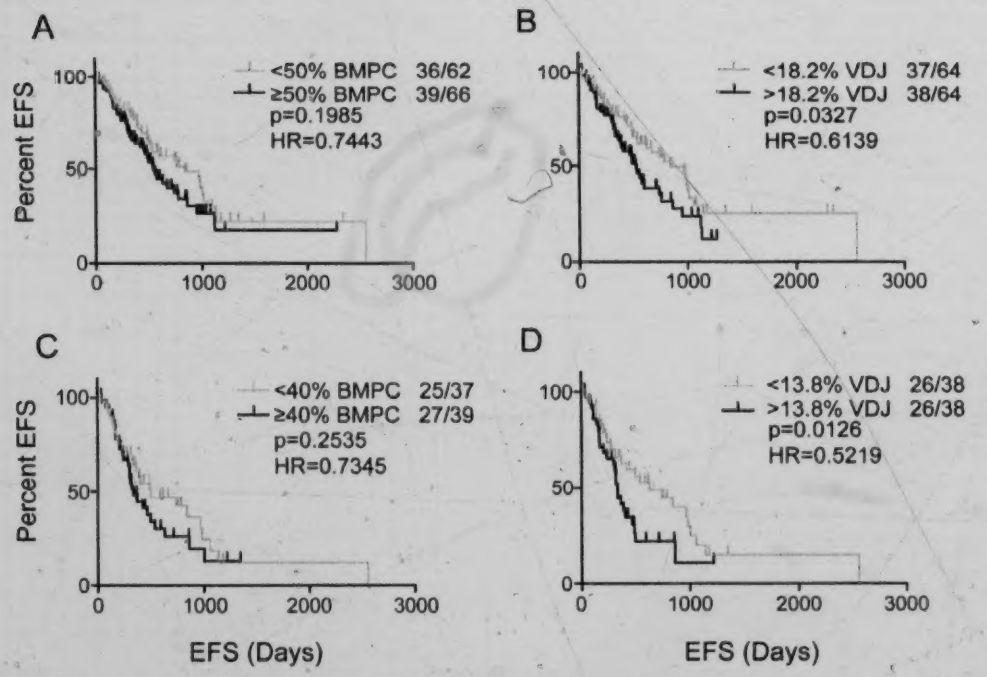


Figure III-9 Kaplan-Meier Curves of BMPC or Matched VDJ% to EFS

The median BMPC percentage dichotomized the group but did not indicate significantly different EFSs (A). The same patient cohort, categorized by VDJ% above and below the median, significantly correlates with EFS (B). If the ASCT treated patient samples are removed, the EFS compared to the BMPC remains insignificant (C) and the VDJ% remains significant (D).

G. Flow cytometry to enumerate BMMC PCs

In this study, the BMPC did not correlate with EFS while the pre-treatment VDJ% does. The types of cells enumerated by each method are different. VDJ% measures all clonal cells and BMPC measures only the PCs. However, these two techniques also generate a percentage using different backgrounds of non-clonal cells. The clinical measure, BMPC, calculates the percentage of PCs among total white cells on a histological smear from a BM aspirate, whereas the VDJ% evaluates the number of clonal cells among purified mononuclear cells. It is important to determine whether the different between VDJ% and BMPC in correlating with EFS is a result of these different backgrounds or in the different types of cells enumerated. To evaluate this, flow cytometry was used to determine the percentage of PCs (PC%) in purified BM mononuclear cells. Here, BM samples that had been previously analyzed with flow cytometry were reanalyzed. Because these samples were processed over a span of about 10 years, there was no consistent staining protocol to measure the number of PCs. The raw data from 47 tests were reanalyzed to apply consistent gating parameters.

Two types of parameters were used. The parameter to include only live cells represents the highest possible PC% (i.e. less total events to PC events) by setting the staining gates to include only those events that fall under a stringent live-cell forward and side scatter gate. This parameter excludes any events that arise from particulate matter or dead cells that would overestimate the total number of cells. However, this parameter may also exclude dying cells that still carry genomic DNA that would be included in an RQ-PCR. The ungated parameter represents the lowest possible percent of PCs by including all forward and side scatter events: live cells, particulate matter and dead/dying cells. The ungated PC% was on average 1.2 times less than the live-cell PC%, with a standard deviation of 0.092.

The PC% from FACS analysis was performed to explore the relationship between this enumeration and the VDJ%. The log differences between the VDJ% values and the FACS analysis PC% values are shown in Figure III-10. Sixty three percent (33/52) of the samples analyzed had a lower PC% than VDJ%. The samples denoted with an asterisk (*) indicate those that lacked a single cell VDJ PCR to determine the proportion of PCs that were clonal.

The VDJ% exhibited a significant, yet modest correlation with the PC% generated by both live-gated and ungated parameters using Pearson's correlation (Figure III-11). In a Kaplan-Meier curve analysis, no significant correlation was observed between the PC% from either parameter and EFS (Figure III-12A and C) or BMPC (Figure III-12D). In contrast to the results seen in sections III.C, III.E and III.F, when the VDJ% values from the same patient set were used in a Kaplan-Meier analysis, there was no significant correlation with EFS. This means that no conclusions can be drawn about that relative value of BMPC versus VDJ% for this subset of patients.

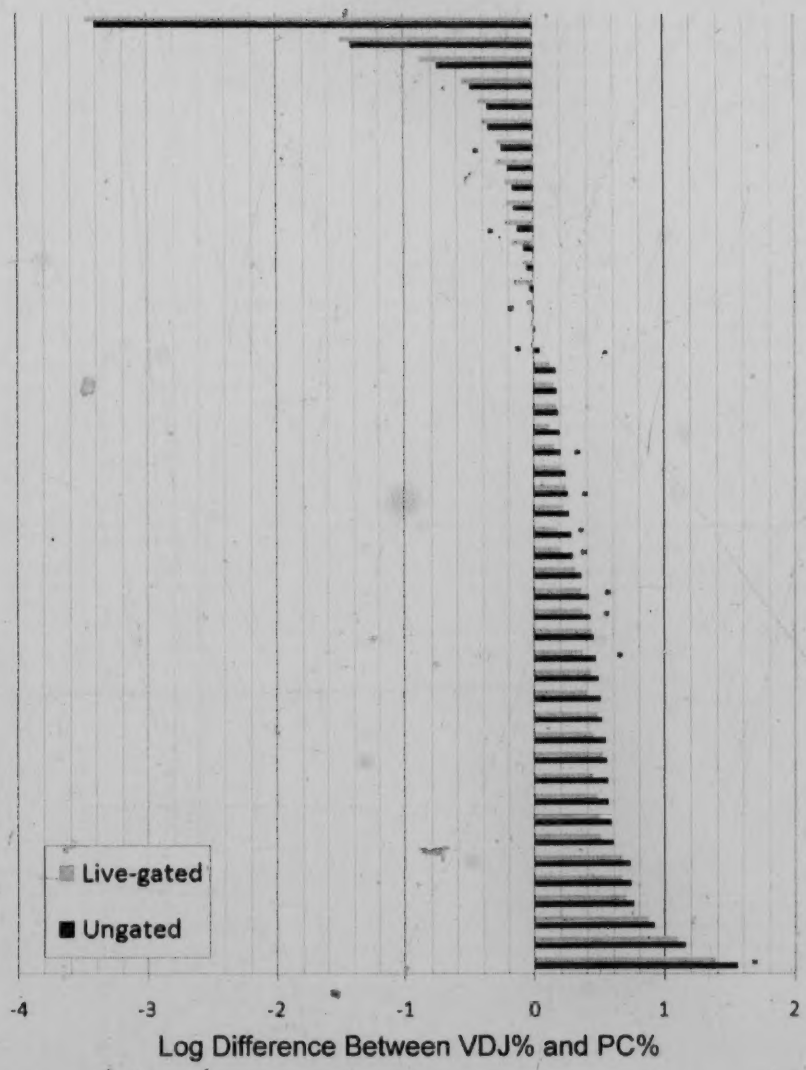


Figure III-10 Log Difference Between the VDJ% and the PC%

The VDJ% was determined using the SG RQ-PCR and the PC% was determined using flow cytometry. The log difference was calculated as the log (VDJ% ÷ PC%). The further the difference is from zero the greater the difference between the PC% and the VDJ% values. A negative number indicates samples with a higher PC% value than VDJ% value. The light bars represent the live-gated parameter and the dark bars represent the ungated parameter. *= samples that did not have a corresponding single cell VDJ PCR test to identify the percent of PCs that are clonal

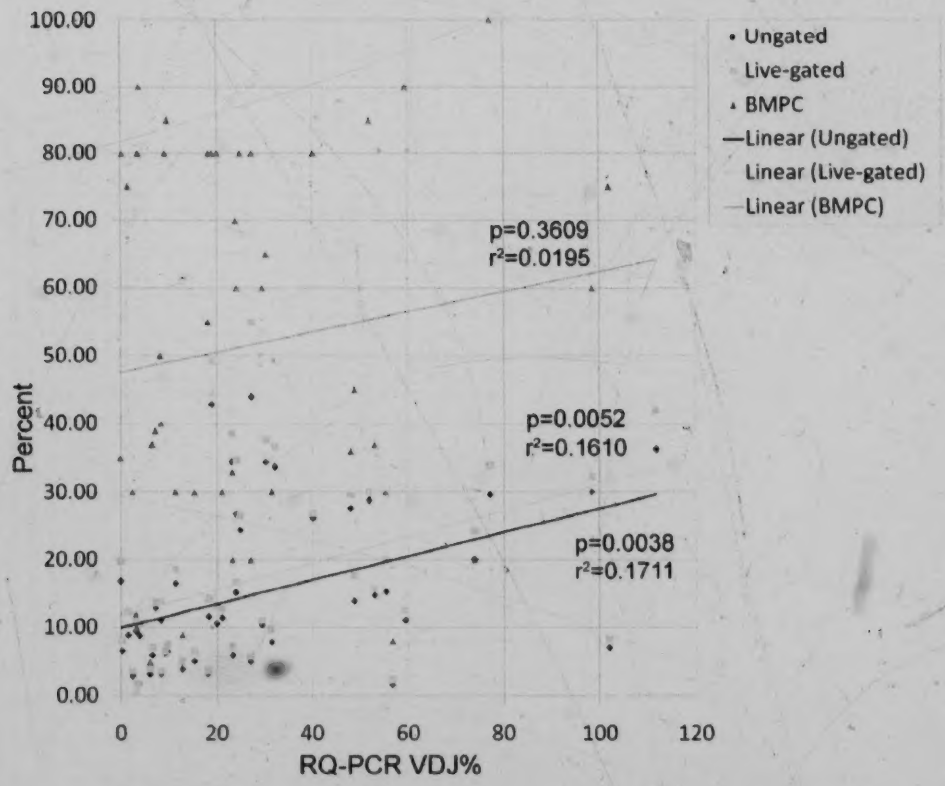


Figure III-11 Correlations Among the RQ-PCR VDJ%, PC% and the Clinical BMPC

The VDJ% was determined from the SG RQ-PCR system and plotted against the matched PC% from the live-gated parameter (◆), the PC% from the ungated parameter (■) and the BMPC percentage (▲). The p-values were determined using a Pearson's correlation. The lines represent the linear regression between the data sets.

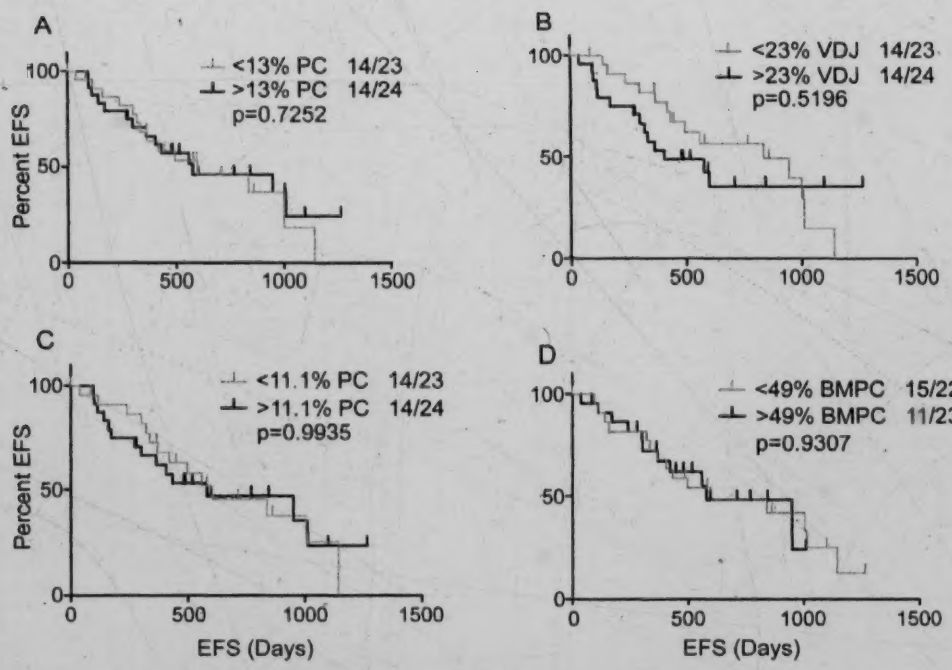
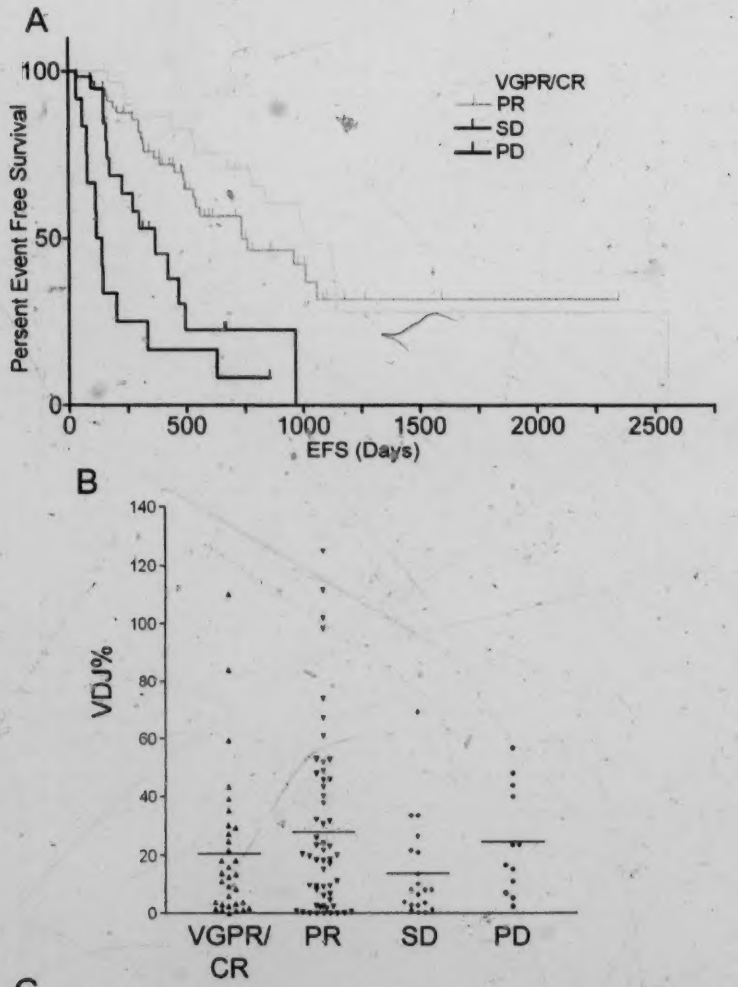


Figure III-12 Kaplan-Meier Analysis of EFS and PC%, BMPC and VDJ%

EFS does not correlate with the PC% as calculated using live cell gating (A) or the ungated parameter (C) as determined by logrank statistics (p-values). The VDJ% from the matched samples also did not correlate with EFS (B), nor did the BMPC percentage (D). The median served to categorize the data sets in each graph where those falling below the median are represented by the light lines and those samples falling above the medians are represented by the dark lines. The fractions in the legends of each graph depict the number of relapses to total samples per group.

H. Tumour response and VDJ%

To analyze the relationship between the pre-treatment VDJ% and treatment response, the IMWG response subcategory was determined for each associated sample. The subcategories showed an association with EFS (Figure III-13A). Significant differences were observed between each response category with the exceptions being between VGPR/CR and PR and between SD and PD (Figure III-13C). However, when the pre-treatment VDJ% values from each response group was evaluated there was no significant association (Figure III-13B and C). In a Fisher Exact test between the pre-treatment VDJ% (dichotomized using the median) and response (dichotomized into PR or better versus SD or worse), there was no significant correlation (p=0.302). Additionally, the median VDJ% of each response subcategory did not vary significantly in a Kruskal-Wallis test (p=0.2415). A Fisher Exact test showed that both the remission VDJ% (p=0.021) and times reduction (p=0.047) were moderately associated with the IMWG response subcategories (where the response was dichotomized into VGPR/CR versus PR).



C

	VGPR/CR vs PR	VGPR/CR vs SD	VGPR/CR vs PD	PR vs SD	PR vs PD	SD vs PD
Logrank: Compared to EFS p=	0.287	<0.0001	<0.0001	0.002	<0.0001	0.287

Figure III-13 Response Subcategories Compared to EFS and Pre-Treatment VDJ%

(A) A Kaplan-Meier curve depicting the EFS of patients classified into four response subcategories with the logrank statistics in C showing a correlation between outcome and response to treatment. (B) The VDJ% values of the patients in different response subcategories, the line represents the mean VDJ% for each grouping. CR=complete response, VGPR=very good partial response, PR=partial response, SD=stable disease, PD=progressive disease.

I. Pre-treatment peripheral blood VDJ%

MM is defined as a cancer of the bone marrow; however, tumour cells have been identified in the PB. It is important to understand the relationship between the BM clonal population and the PB clonal population. Forty-eight pre-treatment PB samples, of which 27 were from relapsed patients and 21 from previously untreated patients, were processed for clonal enumeration by RQ-PCR. A comparison between the VDJ% of the PB and the time-matched BM had an r^2 of 0.431 (Figure III-14) but had conflicting correlation data. Using a Pearson's correlation the PB VDJ% values and the BM VDJ% values were not significantly correlated (p=0.88), while in a Spearman's correlation and Fisher Exact test, the two were significantly linked (p=0.0007, p=0.0002, respectively). Some of the PB samples were negative in the RQ-PCR system and thus given 0.0001% as a value. These numbers were excluded from calculations and Figure III-14; their inclusion does not significantly alter any of the p-values.

In a Kaplan-Meier curve analysis, where the median served to dichotomize the upper and lower PB VDJ% values, the logrank test showed no significance between the EFSs of the two groups (p=0.2656). When the dichotomizing value was adjusted, significant differences in EFSs emerged. At a value of 0.22%, the p-value was the lowest (Figure III-15). This type of analysis is equally as valid as categorization by median; it simply means that there are not an equal number of samples in each grouping.

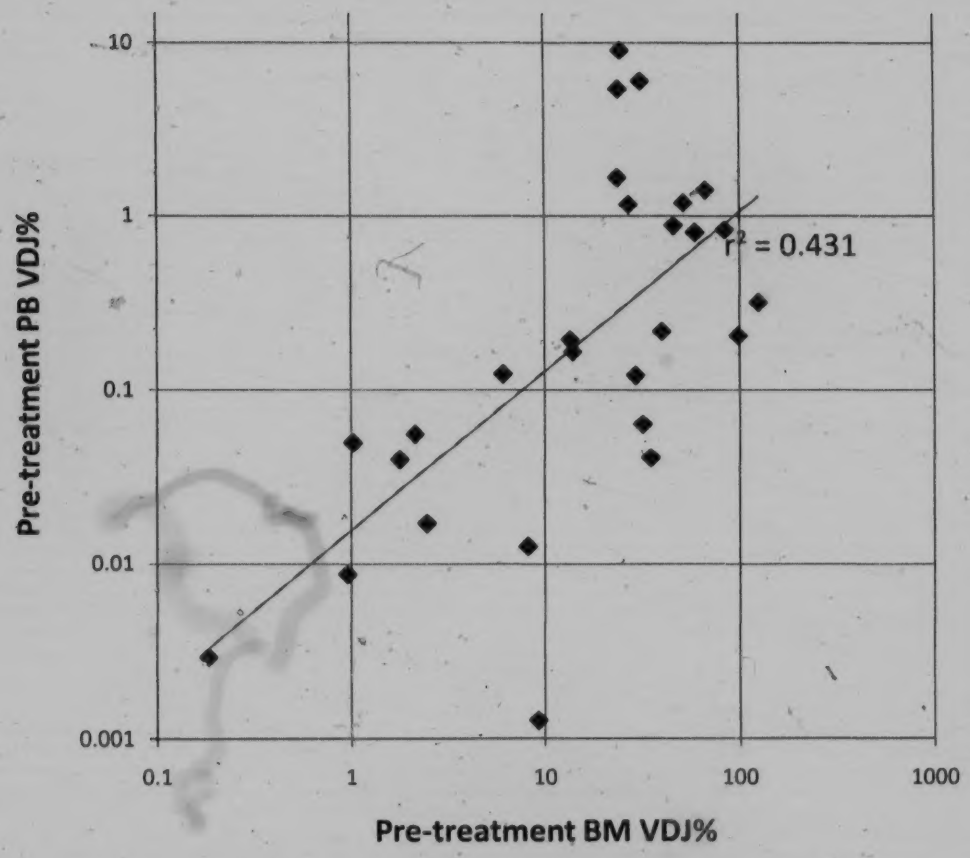


Figure III-14 Correlation Between the Pre-Treatment BM VDJ% and PB VJD%

The pre-treatment VDJ% from BM samples are plotted along the x-axis and the time-matched PB samples are plotted along the y-axis. The line represents the linear regression used to calculate the r^2.

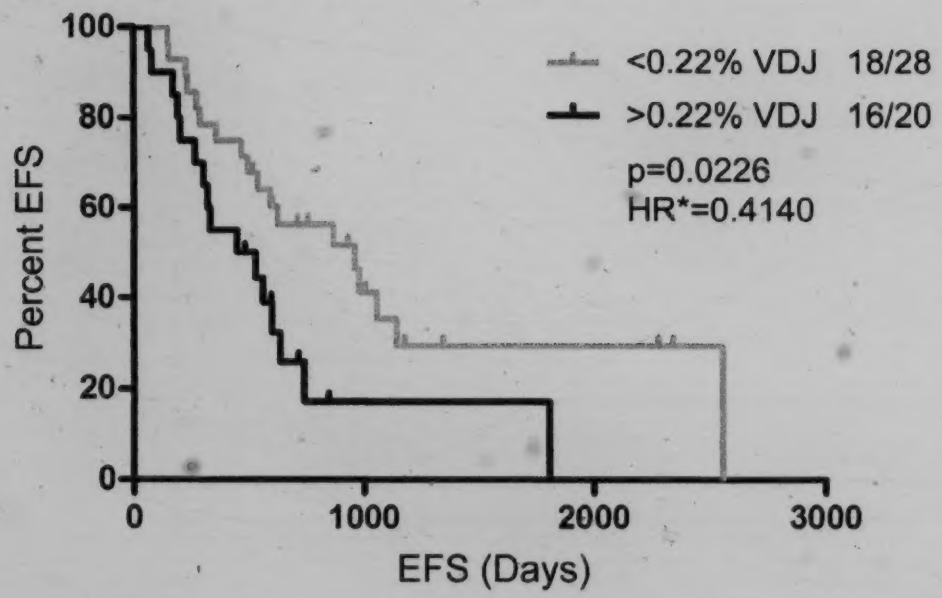


Figure III-15 Kaplan-Meier Curve of EFS and Pre-Treatment PB VDJ%

This Kaplan-Meier curve shows a significant association between EFS and the pre-treatment PB VDJ% where those patients with lower VDJ%s (light line) have longer EFSs than those with higher VDJ%s (dark line). In the lower VDJ% group, there were 18 relapses in 28 patients compared to 16 in 20 from the upper VDJ% group. The p-value was calculated with a logrank statistic.

*HR=hazard ratio

J. Post treatment initiation PB VDJ%

To assess the efficacy of a treatment, patients return to the clinic regularly to have the levels of various markers evaluated. It is essential to understand whether the kinetics of the tumour elimination from the PB as measured here can also predict outcome. Two sets of measures were evaluated here, post-treatment initiation VDJ% and the times reduction comparing the post treatment initiation VDJ% to the pre-treatment VDJ%. Patients were followed every month for the first four months after treatment initiation. The Kaplan-Meier curve analysis comparing EFS and times reduction in tumour burden from months 1 through 4 are seen in Figure III-16A, D, G and J and did not indicate any significant associations. In a Kaplan-Meier analysis of the VDJ% from these monthly samples, where the median dichotomized the groups, there was no significant correlations with EFS (Figure III-16B, E, H and K). When the dichotomizing values were adjusted in the VDJ% analysis significant associations were observed for month 1, month 2 and month 3 but not for month 4 (Figure III-16C, F and I).

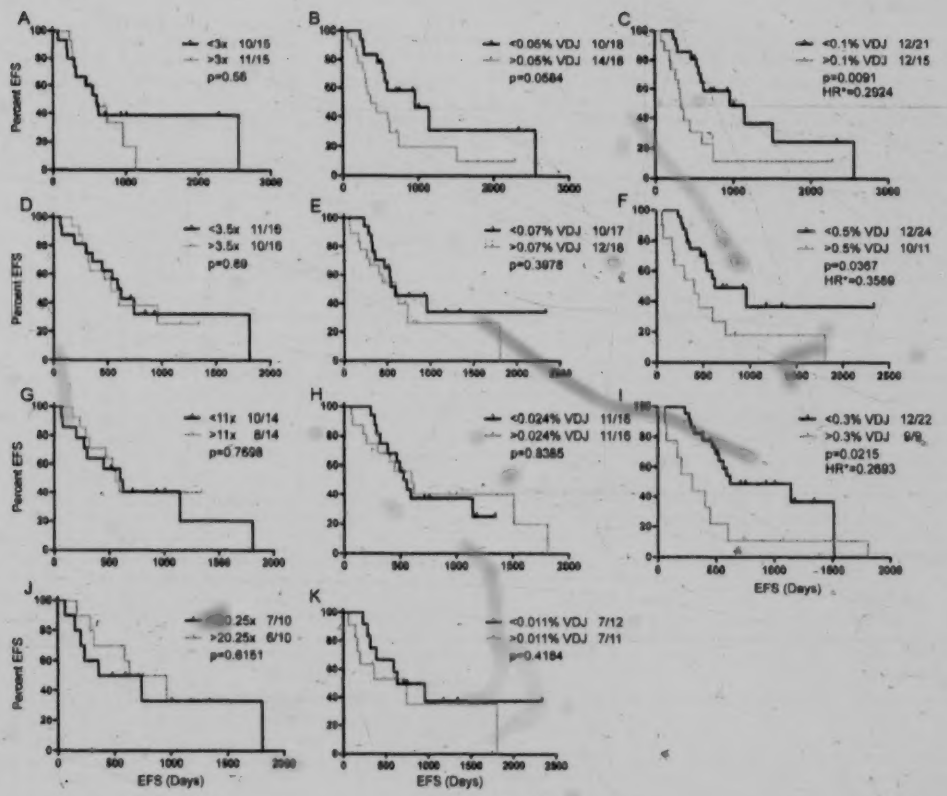


Figure III-16 Kaplan-Meier Curves of PB in the First Four Months After Treatment Initiation Versus EFS

The times reduction of the VDJ% from pre-treatment PB after month one (A), month two (D), month three (G) and month four (J) do not significantly correlate with EFS. The median times reduction dichotomized each group. The VDJ% from month one (B), month two (E), month three (H) and month four (K) after treatment initiation also do not correlate with EFS. The median VDJ% dichotomized each of these groups. When the VDJ% dichotomizing values were adjusted, significant correlations were observed for month one (C), month two (F) and month three (I) after treatment initiation. The p-values were determined using the logrank statistic. The fraction in each legend represents the number of relapses over the number of total samples included.

*HR=hazard ratio

IV. Discussion

A. SYBR green sensitivity

The RQ-PCR system using SG had a lowest level of detection at 0.001%. This was determined by a dilution assay in which 2 of 3 replicates of normal PBMC DNA spiked with cell line DNA to 0.001% were positive in a SG RQ-PCR test. Previously suggested, 0.001% is the required minimum level of detection for accurate quantification of tumour cells in a MM patient sample (145).

A single target sequence amplified in an RQ-PCR reaction would result in a C(T) value of 35, as calculated by reversing the aggregate curve slope formula: C(T)=((log*1molecules*/log10)-9.708)/ -0.2774). In the RQ-PCR reactions performed here, the C(T) values for the VDJ reactions of the 0.001% dilution were 35.12 and 34.84, which is calculated to 1.01 molecules. In one study using SG RQ-PCR in MM, a detection limit of a single target was also observed (137).

The percent of clonal cells calculated from this dilution using the β2m RQ-PCR results as a reference, was 0.0025%. While this percent is 2.5 times different than the target percent, 0.001%, it equates to a C(T) difference of 1.4, within the variation allowable between RQ-PCR replicate reactions (139). The acceptance of 0.001% as the lowest limit of detection can be deemed acceptable.

B. LDA quantitative PCR

The LDA has long been used to determine the clonal tumour burned in MM. It is used here to confirm the results of the SG RQ-PCR. In the BM and PB samples, the LDA generated VDJ% values were four one hundredths of a percent higher than the VDJ% values calculated from the RQ-PCR. While the sample size is low, only 2 samples, the clonal percentages

calculated were very similar and indicate that the VDJ% generated for the SG RQ-PCR system can be reliable.

C. Pre-treatment BM VDJ% correlates with EFS but not OS

The percent of clonal cells measured by RQ-PCR in aspirated pre-treatment BMMCs significantly correlated with EFS in a Kaplan-Meier analysis. Samples from patients receiving chemotherapy or biologically based therapy, but ineligible for an ASCT, continued to correlate with EFS, while samples from patients receiving an ASCT did not. Subsequently, three significantly different remission durations were revealed. Patients receiving an ASCT had the longest remission duration; patients not eligible for an ACST with a VDJ% below 17% (the median in this data set) had the next longest remission duration. Finally, patients not eligible for an ACST with a VDJ% above 17% had the shortest remission duration in this study. Patients treated with an ASCT are known to have longer remissions than those treated with chemotherapy alone (82, 113).

Although the number of completed endpoints is small and the follow up time is short, VDJ% cannot dichotomize the ASCT treated patient cohort into significantly different EFS groups. More patients may be required to reveal a significant association between the VDJ% values of ASCT treated patients and EFS. In contrast, samples from patients ineligible for an ASCT show a significant association between VDJ% and EFS. Of note, in Figure III-7C, the samples depicted were from patients not treated with an ASCT and show that with many fewer samples, a significant association was still observed.

VDJ% was able to predict outcome when the variables were dichotomized into two and three groups, but not when treated as continuous variables. This has been previously shown

with remission VJD%: no linear correlation with outcome was observed but dichotomizing the remission VDJ% values showed a significant correlated with EFS (153).

A stem cell transplant is the most effective treatment for patients with MM, and the loss of correlation with outcome, compared to the non-transplant group, may further exemplify the efficacy of this treatment. Others have reported the pre-treatment levels of clonal cells (137, 152, 154) but fail to cite any associations between tumour burden and outcome. This may be due to the use of samples taken from patients later treated with an ASCT. As indicated here, the VDJ% for such patients alone does not have prognostic significance. In the current study, however, patients were treated with a variety of therapies with both of front-line and relapsed/refractory myelomas, and may be the reason why this data shows prognostic significance.

It is important to note that most studies identifying EFS prognostic indicators, like serum β2m and albumin (10, 158) levels, chromosome 13 monosomy (159, 160), and t(4;14) translocations (161) analyze samples from previously untreated patients. However, patient can only be 'previously untreated' once, and for the purposes of defining relapse risks for each cycle of therapy these factors are less defined. This study considers all the treatment cycles, as VDJ% is predictive of EFS in untreated and relapsed MM. As part of clinical testing, VDJ% may indicate to a treating clinician the current likelihood of early relapse. The advantage is that this measure adjusts with the evolving malignancy.

Overall survival is the length of time between a patient's diagnosis and death. In the patient sampling here, VDJ% does not correlate with OS. The cohort has a limited follow-up time and includes few complete endpoints (deaths); this may explain the lack of a significant association. Previous studies have shown that the time to first disease progression (first

relapse) correlates with survival (162) and if VDJ% correlates with time to disease progression (EFS) it remains possible that with more sample numbers, VDJ% may correlate with OS. In the survival analysis that includes all samples, time to death (TTD), there also is not a significant correlation between VDJ% and survival. This result may also change with an increase in sample size and follow up time.

LDH and β2m are the only two other parameters analyzed here to show a significant association with EFS. The other parameters fail to correlate to EFS most likely due to the small sample size and diversity of disease stages represented; these parameters have been previously implicated as prognostic indicators of outcome using a large number of frontline treated patients.

D. Remission BM VDJ% does not correlate with outcome

In this study, the VDJ% from remission/post treatment BM samples did not predict outcome. This is in contrast to previously published studies that show the level of clonal cells in even a small number of samples can correlate with EFS. The samples used in each of these studies were obtained from uniformly treated patients and often collected at similar time points. The remission BM samples used in this study were extracted from patients treated with a variety of therapeutic regimens and from varying time points in the disease. Samples were collected from frontline post VAD or bortezomib treated patients and from the remissions of patients receiving lenalidomide, bortezomib, or Dex for relapsed disease. These latter samples were taken at varying time points during remission. As no significant correlation was observed between the remission VDJ% or times reduction and EFS from these unequal time points the TTP was used to standardize the time points of BM sampling. However, VDJ% and times remission still did not correlate with TTP. Irregular sampling may explain the difference in

significance between this data and others. In addition, limited follow-up time and number of endpoints (relapses) may also explain the difference. That there are contrasting results suggest that the significant association between remission clonal levels and EFS should be used with caution. One of these studies only contained data from 20 patient samples (151). In the current study, 32 samples were used, suggesting that more samples may not identify a correlation, and /or that previous results may be valid only in specific situations. As the remission samples studied here were obtained from different treatment regimens for different stages, the data may be more accurate.

If the results here depict the true situation, that the remission BM VDJ% does not correlate with outcome then a model of myeloma growth can be suggested. In pre-treatment BM, the number of clonal cells could proportionally represent the number of progenitor cells. A BM aspirate would contain a number of clonal cells that reflect this proportional population. When the tumour is treated, this representative population is disproportionally lost and no longer reflects the size of the progenitor compartment. Additionally, due to geographical constraints, sampling of the bone marrow during remission may result in a large variation in tumour levels due to non-random distribution of the tumour in the marrow space. As relapse is inevitable, the progenitor compartment escapes therapy and reestablishes the tumour mass.

This is the first report to show the remission clonal levels of BM samples after frontline treatment with bortezomib. It is shown here that the levels of VDJ% and times reduction do not significantly differ between this and the VAD/ASCT treated group, indicating that bortezomib is an equally effective treatment at reducing the disease burden. The remaining samples were from relapsed myelomas and represent patients treated with lenalidomide, bortezomib and Dex and the number of patients are too small for separate interpretation of the results.

E. Aggregate standard curve

The generation of the aggregate standard curve represents an opportunity for rapid clonal quantification in a much larger patient cohort. The cloning of an individual amplicon for a standard curve is a lengthy process. Typically, a fresh amplicon is made, cloned into a vector and transformed into bacterial cells. Colonies are grown, then selected and grown again in liquid culture. Plasmid is isolated from these cultures and tested to ensure the correct sequence was cloned. This process takes 3-4 days because of bacterial growth times. The plasmid is then diluted in a tenfold series and run with each VDJ PCR to generate the specific PCR efficiency. A cloned PCR product is common for standard curves (139, 163). However, without the need to generate cloned amplicons for each patient, analysis using an aggregate standard curve would be faster and the number of RQ-PCR reactions would be greatly reduced. The aggregate curve must be validated to guarantee accuracy. The purpose of the standard curve is to correct the C(T) value with the specific PCR efficiency. A PCR that is 90% efficient will have different C(T) values than a PCR that is only 70% efficient. In general, the aggregate curve was appropriate for most reactions. For 6 of 44 samples the aggregate curve resulted in a switch from low to high VDJ%, and vice versa, based on the medians. However, the Kaplan-Meier curves of the individual and aggregate standard curve generated VDJ% values showed a similar significance association with EFS. Additionally, a Spearman's correlation and Fisher Exact Test proved the VDJ% values were highly correlated and that the values generated from the aggregate curve are acceptable.

The drawback is that the aggregate curve generated VDJ% values are not the 'actual' VDJ% values: a PCR with a reduced efficiency will have greatly different values generated between the individual and aggregate standard curves. Excellent primer design and primer testing reduces the occurrence of poor efficiency PCRs.

F. BMPC, VDJ% and outcome

The BMPC is a clinical measure of PCs in a BM aspirate. It is known that these counts do not correlate with outcome (125). To further understand the value of VDJ% as a predictive marker of EFS, other factors that directly measure tumour burden should be investigated. It was shown that VDJ% was significantly correlated with the BMPC PC%. This association is very weak however, with an r^2 value of only 0.11. This means that only 11% of the variation in the BMPC values can be explained by the VDJ% values, leaving 89% to unknown associations. The VDJ% values are lower than the BMPC values most likely due to the hemodiuted nature of the aspirates used for VDJ% evaluation. Additionally, the BMPC is typically measured from a section of bone marrow with a high concentration of PCs.

In this sample cohort, no association was observed using Kaplan-Meier analysis between the BMPC and EFS. However, for the sample patient cohort, a significant association with EFS was observed using the VDJ% values. The VDJ% from patients receiving an ASCT was not predictive of EFS and this may be true with BMPC as well. However, exclusion of ASCT treated patients from this analysis continued to indicate that BMPC was not associated with EFS.

A hypothesis about the MM growth compartments can be derived from this data. The BMPC only measures PCs without any further clonal identification. On the other hand, the VDJ% counts all cells containing the clonal sequence, regardless of their morphology or differentiation stage. It is possible that the difference between the two clonal measures reflect a population of cells that are counted by the RQ-PCR but remain unobserved by the morphological enumeration of PCs. It can then be postulated that these cells are responsible for the correlation between EFS and VDJ% and the lack of correlation between EFS and BMPC. Further, it can be suggested

that these cells are involved in the expansion of the malignancy; a greater number of progenitor cells leads to a shorter remission.

G. Flow cytometry

As noted in the results, analyzing the BM samples using flow cytometry is a better method for comparing the number of PCs to VDJ%. Having come from the same BMMC sample, correlations here will indicate if the proposed model of MM above (clonal non-PCs are being included in the VDJ% and are biologically relevant in predicting outcome) is possible. If this model is correct, the PC% as determined from flow cytometry will not correlate with EFS but the VDJ% values from the same samples will correlate. The data here reveal that indeed, the PC% did not correlate with outcome but neither did the VDJ% from this cohort. As such, the results are non-informative. Additionally, if the model above is correct, the VDJ% should consistently be higher than the PC%, as the RQ-PCR enumerates the clonal PCs and clonal non-PCs. The VDJ% was higher than the PC% for only two thirds of the samples tested. One third had a higher PC% then VDJ%. A possible explanation for this observation may be that the aggregate standard curve underrepresented the VDJ%. In one study comparing RQ-PCR VDJ% with flow cytometry determined PC%, 6/17 or approximately one third of the samples had a higher PC% then VDJ% (151), individual standard curves were used to generate the VDJ%. Alternatively, the VDJ% may be understated if the number β2m molecules are higher due to a trisomy. Observed by Debes-Marun *et al.* (164), chromosome 15, which contains the β2m gene, occurs in clonal cells as a trisomy in 22% of MM patients. This extra copy of β2m in each clonal cell could influence the VDJ% result by falsely increasing the cellular background used to generate the percent. Additionally, contamination of the single PC clonality PCR might overrepresent the true clonal PC%.

Matching flow cytometry results and VDJ% values were found for 52 samples. It was observed that the PC% from flow cytometry did not correlate with EFS. However, it was also observed that the VDJ% from this cohort also did not correlate with outcome. It would be necessary to include more samples to resolve this issue. If after adding more samples PC% could not predict EFS but the VDJ% could, it would confirm the hypothesis, that the VDJ% is measuring vital compartments of the MM clone that maintain the disease. Similarly, Sarasquete *et al.* were able to predict outcome using remission VDJ% but not remission PC% (151). In contrast, if a larger cohort revealed that both VDJ% and PC% correlated with EFS then the difference between the VDJ% and BMPC in predicting outcome would not be due to the measurement of different populations. Instead, the most plausible explanation would be that VDJ% and BMPC are counting PCs in different non-clonal backgrounds: VDJ% is looking at total BMMC while the BMPC is looking at total white cells.

H. Tumour response and VDJ%

In this study, the tumour response subcategories outlined by the IMWG show some correlation to EFS. The better the tumour responds to therapy, the longer the cancer stays in remission. Additionally, while EFS and VDJ% are correlated, it is shown by multiple statistical examinations that VDJ% and treatment response are not significantly associated. This result suggests that VDJ%, as a prognostic indicator of EFS, may be independent of response. More samples may provide enough statistical power to combine the VDJ% and response into new categories to further delineate outcome, where patients with low VDJ% and a good response have a better outcome then those patients with high VDJ% and good response, for example. This observation also confirms potential independence of treatment type. Although some treatments are claimed to effect more CRs than others, the work reported here shows that the VDJ% predicts outcome prior to treatment choice and efficacy. Tumour response as defined by

the reduction of PCs or other tests has long been used to measure drug efficacy; however, it has been shown that the magnitude of response does not correlate with outcome (83, 162). Indeed, response plays an unusual role in MM. The magnitude of response is not correlated with outcome, but slow tumour reductions and the achievement of a CR can correlate with longer survival (47).

In contrast, the remission VDJ% and times reduction were correlated with response in a Fisher Exact test. Logically, response is based on the amount of tumour reduction as is remission VDJ% and times reduction.

I. Pre-treatment PB clonal cell enumeration

It was shown that the BM VDJ% was predictive of EFS, however the relationship between the BM samples and PB samples is also important. BM aspirates can be painful and are not frequently performed during routine clinical visits. A PB sample is much easier to obtain and could be used to predict outcome. In non-parametric statistical examinations, the pre-treatment levels of BM VDJ% and PB VDJ% were correlated. A Kaplan-Meier analysis showed that pre-treatment PB VDJ% was predictive of EFS using 0.22% VDJ as the dichotomizing value. In this data set, the median was unable to dichotomize the VDJ% values into meaningful groups. Similar to maximally selected log-rank statistics (165), the dichotomizing value was adjusted to find the lowest p-value and thus the most meaningful cutoff. The data here show that the pre-treatment PB VDJ% can also categorize patients into significant groups with differing remission lengths. However, many more samples must be analyzed to confirm these results and to obtain a fuller understanding of PB VDJ% as a prognostic indicator.

J. Post treatment initiation PB clonal cell enumeration

As with the pre-treatment PB VDJ%, no significant association with EFS and the post treatment VDJ% or times reduction from PB samples was observed using the median to divide each data set into equal groups. If the dichotomizing values are adjusted (all higher), as noted for PB pre-treatment VDJ%, then significant associations with EFS were found for the 1, 2 and 3 months post treatment initiation VDJ%. Patients with a higher VDJ% values in their PB sample were more likely to relapse sooner than those with fewer clonal cells. At 4 months, significance could not be found by adjusting the cutoff value, which has at least two explanations. First, the sample size is insufficient to generate significance. Second, the loss of significance may be due to non-random loss of the clonal cells during treatment. Bakkus et.al (153) observed a decrease in the significance of the BM VDJ% from 3 to 6 months post treatment. This observation seen by Bakkus may also represent a non-random loss of cells that proportionally reflect the progenitor population. Losing these cells would lead to a clonal level (VDJ%) that is unable to significantly dichotomize patients into meaningful EFS groups. However, it is important to note that this is a very preliminary study. More samples would be required in order to confirm the results seen here.

K. Future directions

As noted throughout this discussion, additional patient samples would be essential to make more solid conclusions. In particular, additional BM samples would be needed to definitively identify VDJ% as a prognostic marker. Factors that predict outcome are best evaluated by using two separate data sets. The first set of patients is used to generate the best cutoff point and the second is to verify that the cutoff value is significant in another data set. Longer follow-up times and more samples would strengthen the OS curve result; or it may

reveal a significant correlation between pre-treatment VDJ% and OS. The remission samples used in this study were obtained from different time points of remission and from patients treated with a variety of regimens. The results here did not correspond with observations made by others.[1] Similar conclusions may be obtained if the remission samples were from similarly treated patients and/or from a specific time point during remission (i.e. 3 months post transplant. More remission samples would reveal with greater assurance whether or not newer biologically based treatments result in similar VDJ% responses. A further study would be to investigate patients with MGUS or smoldering myeloma for VDJ% correlations, to ask whether RQ-PCR can reveal biologically relevant populations in these diseases. The study of PB would require many more samples to confirm or deny the observations seen here. As noted above, a practice and test group would better identify a prognostic factor.

One possible conclusion from the results here is that clinically relevant, non-PC populations are responsible for the correlation with EFS, while other clinical measures fail to correlate. To further this idea, sorted non-PC and PC compartments could be enumerated for clonal cells and tested for correlations with outcome, revealing if any of these subpopulations individually contribute to disease.

A further step would be to investigate the causes behind this correlation. It could be theorized that the difference between high VDJ% samples and low VDJ% samples is increased proliferative activity of the tumour. Differences in progenitor cell proliferation/expansion could explain the VDJ% and remission length diversities. Myelomas with high replication potential would have a high VDJ% at disease height and a shorter EFS (it would take less time to re-grow the tumour mass). This problem could be analyzed by investigating the relationship between VDJ% and the growth capacity of the various compartments in a 3D MM growth culture as

developed by Dr. Kirshner of the Pilarski lab. It would be expected that the proliferation of the PC and B-cell compartment would be greater in samples with high VDJ%, if the assumptions are true.

L. Global implications and conclusions

The data presented here has the potential in influence the way MM is staged and treated. This is the first report to suggest and observe the pre-treatment BM VDJ% as a prognostic marker for relapse. Of particular importance is that the pre-treatment patient cohort was treated with a wide variety of therapeutic regimens with different responses. The prognostic significance is independent of response and treatment type. The possible exception to this is ASCT. In samples from patients later treated with an ASCT, no further subdivision significantly categorized patients into different outcomes. However, additional samples may show EFS differences in patients treated with different therapies. Many of the prognostic factors currently used by clinicians to evaluate the severity of MM are surrogate markers for tumour burden. VDJ%, however, is a direct measure of the aggregate populations that comprise the tumour and thus may represent the most valuable method for disease prognosis.

For predicting relapse times in patients, pre-treatment VDJ% was highly significant. A patient's VDJ% status, high or low, could be determined for each treatment cycle during a disease course and provide information about the probable length of remission. VDJ%, as determined by SYBR Green I in an RQ-PCR system, has been shown here to be highly accurate and very sensitive. Aggregate curves can be generated to increase screening efficiencies but at the price of individual VDJ% accuracy. It is postulated that VDJ% reveals an important population of clonal cells not identified by other clinical measures. These populations could be a part of the MM growth compartment and progenitor population, and are clinically relevant. The

same enumerated population can be observed in pre-treated PB samples and lost during therapy.

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